INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Quadros et al.
Journal/Book Title:	Biochemical and Biophysical Research Communications
Article Title:	
Volume (Issue):	222 (1)
Pages:	149 - 154
Year of Publication:	1996.
Publisher:	
Remarks:	

NPL

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 222, 149–154 (1996)
ARTICLE NO. 0713

Characterization of Monoclonal Antibodies to Epitopes of Human Transcobalamin II

Edward V. Quadros,[1] Sheldon P. Rothenberg, and Patricia McLoughlin

The Department of Medicine, Division of Hematology/Oncology, SUNY-Health Science Center, Brooklyn, New York 11203; and the Veterans Affairs Medical Center, Brooklyn, New York

Received March 21, 1996

Cellular uptake of cobalamin (Cbl) is mediated by transcobalamin II (TCII), a Cbl binding protein in the plasma. The TCII-Cbl complex binds to a cell surface receptor and is internalized by endocytosis. We have generated monoclonal antibodies (mAbs) to human TCII that can be distinguished into three functional types on the basis of interaction with three different regions of the protein. Type 1: Receptor blocking. This mAb binds holo-TCII and inhibits the cellular uptake of Cbl. Type 2: Cbl blocking. This mAb binds apo-TCII at or near the Cbl binding domain and inhibits the formation of holo-TCII. Type 3: Precipitating. This mAb binds both holo-TCII and apo-TCII but does not interfere with Cbl binding. Whereas type 1 and type 2 mAb, following incubation with TCII-[^{57}Co]Cbl or apo-TCII, respectively, inhibit the uptake of radio-labeled Cbl by K562 cells, type 3 mAb has no such activity with either form of TCII. These properties of type 1 and type 2 mAb that inhibit the cellular uptake of Cbl, may serve to induce rapid Cbl deficiency and provide a model to study the effect of selective Cbl depletion on cell division and differentiation as well as on the pathways dependent on the two Cbl cofactors, methyl-Cbl and 5′-deoxyadenosyl-Cbl.

Transcobalamin II (TCII) is a 45 kDa plasma protein that binds cobalamin (Cbl) during assimilation in the distal ileum (1) and then transports and promotes the cellular uptake of the vitamin by endocytosis via receptors on the plasma membrane for TCII-Cbl. TCII is synthesized primarily by vascular endothelium (2) and circulates predominantly as apo-TCII because holo-TCII is cleared rapidly from the plasma with a half-life of ~90 minutes (3).

Cellular deficiency of Cbl impairs two metabolic pathways: 1. molecular rearrangement of methylmalonyl-CoA to succinyl-CoA which requires 5′-deoxyadenosyl-Cbl as the cofactor (4); and 2. methylation of homocysteine to form methionine and this requires both the methyl-Cbl cofactor and the N^5-methyltetrahydrofolate substrate that provides the methyl group for homocysteine (5). This transfer of the methyl group also regenerates the tetrahydrofolate pool. In Cbl deficiency, methyl-Cbl is depleted "trapping" N^5-methyltetrahydrofolate which, in turn, reduces the pool tetrahydrofolate from which the folate cofactors required for nucleic acid synthesis are derived (6). The clinical consequence of the perturbation of these pathways in humans is megaloblastic hematopoiesis often accompanied by functional and structural disorders of the nervous system (7).

There have been a number of reports of antimetabolites which impair the intracellular function of Cbl cofactors (8). The deleterious effect of nitrous oxide on methionine synthase (9) and on the synthesis of methyl Cbl (10) has been established experimentally and observed clinically with the development of megaloblastic erythropoiesis following exposure to this anesthetic (11). These observations establish the essential role of Cbl in normal cell replication and provide a rational basis for inducing Cbl depletion as a strategy to impair cell replication. Toward that end, we have generated monoclonal antibodies (mAb) to TCII which block the binding of TCII-Cbl to the TCII receptor on the plasma membrane and impair the cellular uptake of this essential vitamin. In this report, we describe the immunoreactive and functional properties of these antibodies.

[1] Corresponding author. Fax: (718) 270-1578. E-mail: quadros@medlib,HSC.Bklyn.edu.

0006-291X/96 $18.00

METHODS

TCII was purified from Cohn fraction III of human plasma as previously described (12) and apo-TCII was prepared by denaturation and dialysis of holo-TCII (13). BALB/c female mice were immunized with 10 μg of a mixture of holo and apo-TCII followed by 2 booster injections of 10 μg at two week intervals and the splenocytes were fused with mouse myeloma NS-1 cells. The hybridomas generated were screened for antibody to holo-and apo-TCII using the ELISA plate assay. Positive supernates in the ELISA assay were further screened for epitope specificity.

Preparation of TCII saturated with [^{57}Co]Cbl. Partially purified TCII (12) was incubated at room temperature in 1 ml of Tris buffered saline (TBS) with sufficient [^{57}Co]Cbl to saturate the binding capacity. After ~30 min incubation, free [^{57}Co]Cbl was removed by the addition of 0.5 ml of a suspension of hemoglobin coated charcoal (14). The mixture was vortexed and the charcoal pelleted by centrifugation. The supernatant fraction was collected and used as the [^{57}Co]Cbl labeled TCII in the assay.

Immunoprecipitation of holo-TCII. The immunoreactivity of [^{57}Co]Cbl-TCII was determined by adding 1 μl of hybridoma medium to ~5,000 cpm (15 pg Cbl) of TCII-bound [^{57}Co]Cbl in 500 μl TBS followed by incubation for 2 hr at 4°C. Antibody bound TCII-[^{57}Co]Cbl was determined by the addition of 5 μl of rabbit anti-mouse antiserum followed in 60 min by the addition of 50 μl of a 10% suspension of Omnisorb membranes (Calbiochem). After mixing for 10 min at 4°C, the membranes were pelleted at 15,000 ×g for 15 min, washed once with 1 ml TBS /1% Triton X-100 and the radioactivity in the pellet determined.

Indentification of mAb which blocks the binding of [^{57}Co]Cbl by TCII. Partially purified TCII was titrated to bind approximately 7–8pg of [^{57}Co]Cbl. This diluted apo-TCII was incubated with 1 μl of hybridoma medium in 500 μl of TBS for 2 hr at 4°C followed by the addition of 15 pg of [^{57}Co]Cbl and the incubation continued for an additional 30 min. Free [^{57}Co]Cbl was removed by adsorption to hemoglobin coated charcoal as described above. The radioactivity in the supernatant fraction, representing TCII-[^{57}Co]Cbl, was determined in a gamma counter. A control contained either no mAb or unrelated hybridoma medium. The decrease in the binding of [^{57}Co]Cbl to TCII in samples containing mAb compared to the control was computed as percent blocking.

Identification of the mAb which blocks the binding of TCII-[^{57}Co]Cbl to the TCII receptor on the plasma membrane. K562 cells from a 48–77 hr suspension culture in Dulbecco's minimal essential medium (DMEM) and 10% fetal bovine serum (FBS) were washed in Hank's balanced salt solution containing 1% bovine serum albumin (HBSS/1% BSA) and resuspended at a density of 2 × 10^6/ml. A 0.5 ml aliquot of this suspension was added to 0.5 ml of HBSS/1% BSA containing 15 pg [^{57}Co]Cbl-TCII that was preincubated for 2 hr with 2 μl of hybridoma medium. This cell suspension was incubated at 37°C for 60 min, pelleted at 1000 ×g for 5 min, washed once with 1 ml of cold HBSS/1% BSA and the radioactivity in the pelleted cells determined. Control samples contained no mAb or contained medium from an unrelated hybridoma. The blocking effect of the mAb on the binding of TCII-[^{57}Co]Cbl to the cell membrane was computed as the percent decrease in membrane bound radioactivity compared to the control samples.

Effect of the mAb on the uptake of [^{57}Co]Cbl by K562 cells. K562 cells (0.2 × 10^6) were seeded into 2 ml of DMEM/10% FBS contained in each well of a 12 well plate. The effect of the mAb was determined using two Cbl concentrations: 1. the addition of the cell suspension to wells containing 880 pM of [^{57}Co]Cbl bound to TCII and preincubated with the mAb overnight at 4°C with 200 μl of hybridoma medium; 2. the addition of the cell suspension to apo-TCII that was preincubated overnight at 4°C with 100 ml hybridoma medium followed by the addition of [^{57}Co]Cbl for a final concentration of 370 pM. The plates were then incubated at 37°C and cell viability determined by trypan blue exclusion, and cell number, enumerated using a hemocytometer, were assessed at 24, 48 and 72 hr. The cells were then pelleted by centrifugation at 1000 ×g for 5 min, washed twice with 2 ml of cold HBSS/1% BSA and the radioactivity determined in the cell button. Control samples lacked mAb or contained unrelated hybridoma medium.

RESULTS

Primary screening of the medium from hybridomas using the ELISA plate assay identified ~100 clones that reacted with apo- and/or holo-TCII. The epitope specificity and functional properties of 12 clones with an apparently high titer of mAb were then further characterized. As shown in Figure 1, all 12 mAb(s) characterized immunoprecipitated TCII-[^{57}Co]Cbl. However, only 6 of these blocked the binding of [^{57}Co]Cbl to TCII (Figure 2, clones 5–18, 1–6, 1–9, 2–6, 3–5, and 3–9). Of the remaining six mAb(s), three blocked virtually all of the TCII-[^{57}Co]Cbl binding to the TCII receptor on K562 cells (Figure 3, clones 2–2, 3–11, and 4–7). Five of the mAb(s) that blocked the binding of [^{57}Co]Cbl to TCII (shown in Figure 2) also decreased to some extent the binding of TCII-[^{57}Co]CBl to TCII (shown in Figure 2) also decreased to some extent the binding of TCII-[^{57}Co]Cbl to the TCII receptor when preincubated with holo TCII (Figure 3, clones 5–18, 1–6, 1–9, 2–6, and 3–9). The three mAb(s) that immunoprecipitated TCII-[^{57}Co]Cbl (clones 1–2, 1–12, and

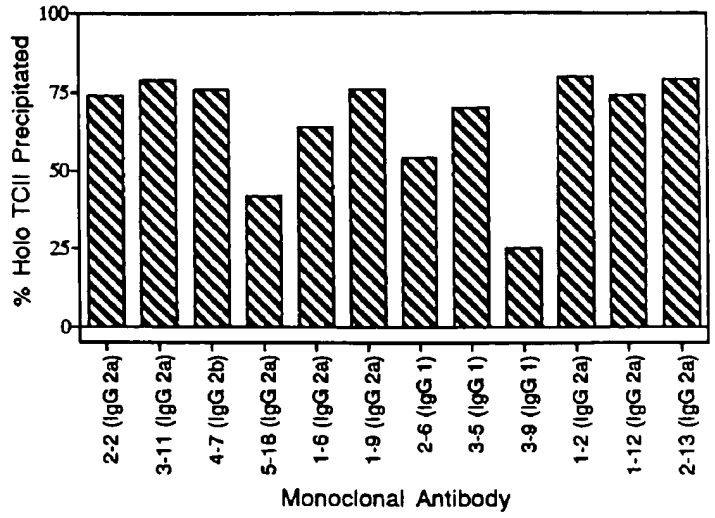


FIG. 1. Immunoprecipitation of TCII-[^{57}Co]Cbl by the mAbs. Each mAb is identified by the designated clone number and the specific immunoglobulin subtype is shown in parentheses.

2–13 shown in Figure 1) neither blocked the binding of Cbl to TCII (Figure 2) nor the binding of TCII-[^{57}Co]Cbl to the TCII receptor (Figure 3).

These 12 mAb(s) are grouped into three types based on their epitope specificity and effect on the functional properties of TCII (Table 1). All three types will immunoprecipitate TCII-[^{57}Co]Cbl but the epitope on TCII for type 1 is in apparent close proximity to the TCII receptor binding site and that for the type 2 is located close to the Cbl binding site. The epitope for type 3 is apparently located at a distance from these two functional regions of TCII because this mAb will not block either the binding of Cbl to apo-TCII or the binding of the complex to the TCII membrane receptor. Since the type 1 and type 2 mAb do not have overlapping immunoreactive specificity, it is likely that the two functional domains of TCII (i.e. Cbl binding and receptor binding) are well separated.

The type 1 and type 2 mAb, in addition to their common functional property of inhibiting TCII-Cbl from binding to the TCII receptor on the cell membrane, also inhibit the cellular uptake of Cbl. Figure 4 shows uptake of [^{57}Co]Cbl-TCII by K562 cells during 72 hr in culture. Four of the Cbl-blocking antibodies tested inhibited Cbl uptake by 75–90% during the culture period. A

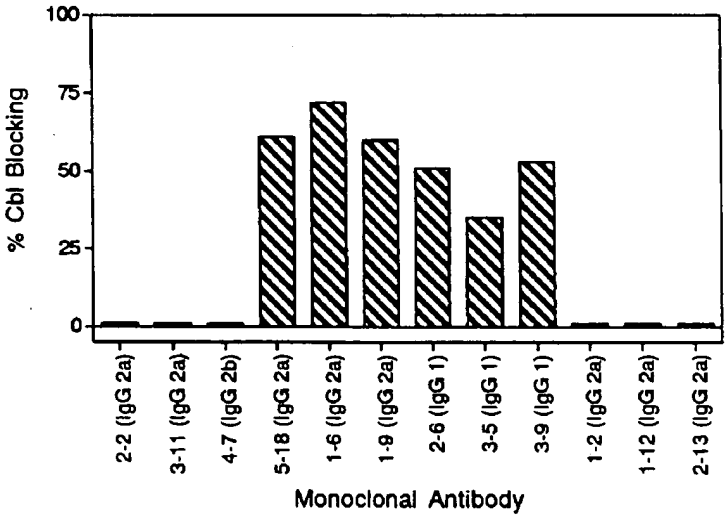


FIG. 2. The blocking effect of the mAb(s) on the binding of [^{57}Co]Cbl by TCII. Apo-TCII was preincubated with the mAb before the addition of [^{57}Co]Cbl. The percentage blocking was calculated from the decrease in the binding of the [^{57}Co]Cbl observed with the mAb as compared to the binding observed in the control lacking the anti-TCII mAb.

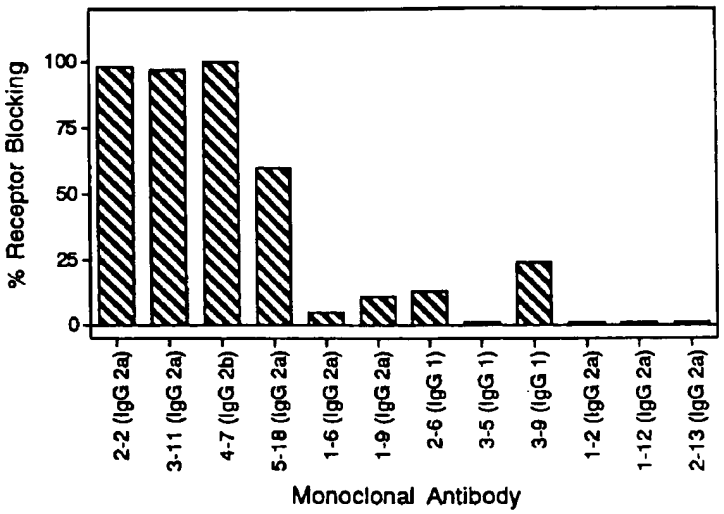


FIG. 3. Blocking of TCII-[^{57}Co]Cbl binding to the TCII receptors on K562 cells by the mAb. TCII-[^{57}Co]Cbl was incubated at 37°C with K562 cells in HBSS containing Ca^{++}. Duplicate tubes containing 10 mM EDTA were used to determine non-specific binding. The decrease in TCII-[^{57}Co]Cbl binding to the TCII receptor is expressed as a percentage of the TCII-[^{57}Co]Cbl bound to cells in the control that did not contain the mAb.

type 3 mAb had no such effect though it did bind apo and holo-TCII. The remaining two type 2 mAbs similarly blocked the uptake of [^{57}Co]Cbl-TCII over 72 and 96 hr (data not shown). The three receptor blocking (type 1) mAbs also inhibited by 25–50% the cellular uptake of [^{57}Co]Cbl (Figure 5). It should be noted that in both experiments the inhibition by type 1 and type 2 mAb of Cbl uptake (Figures 4 and 5) persisted throughout the 72 hr culture period.

DISCUSSION

In this report we describe the immunoreactive and functional properties of three types of mAb(s) generated against human TCII. The type 1 mAb blocks the binding of the TCII-Cbl complex to the TCII receptor on the plasma membrane of K562 cells and the type 2 mAb blocks the binding of Cbl to TCII. The type 3 mAb neither blocks the binding of Cbl to TCII nor the binding of the TCII-Cbl to the TCII membrane receptor although it immunoprecipitates the TCII-Cbl complex.

A significant property of the type 1 and type 2 mAb is that they both block the cellular uptake

TABLE 1
Epitope Specificity and Functional Property of the Monoclonal Antibodies to TCII

mAb	Designation	Epitope	Functional property
Type 1	2–2 3–11 4–7	In proximity to receptor binding site	Inhibits cellular uptake of TCII-Cbl
Type 2	1–6 1–9 2–6 3–5 3–9 5–18	In proximity to Cbl binding site	Blocks binding of Cbl and Inhibits cellular uptake of TCII-Cbl
Type 3	1–2 1–12 2–13	Distant from receptor or Cbl binding site	Does not inhibit cellular uptake of TCII-Cbl

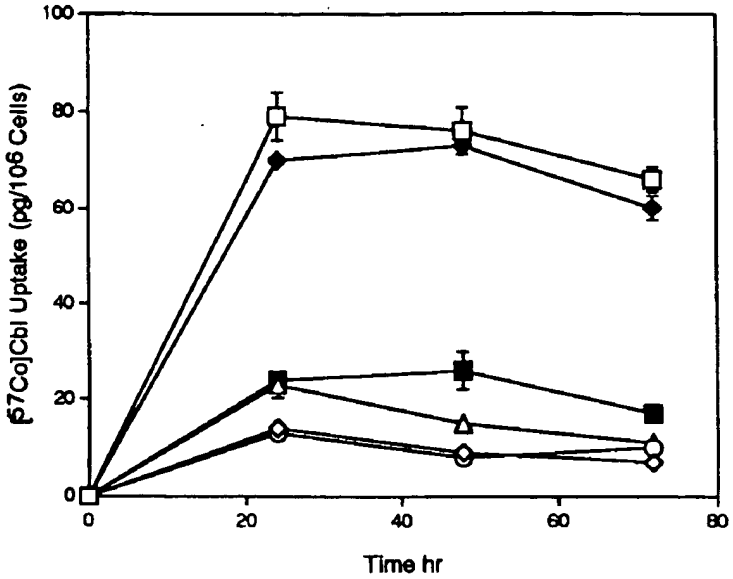


FIG. 4. Inhibition of Cbl uptake in K562 cells by type 2 (Cbl blocking) mAb. Apo-TCII was incubated with the mAb overnight and the mixture added to the cells in culture with sufficient [^{57}Co]Cbl to saturate the binding capacity of TCII. The uptake of [^{57}Co]Cbl by the cells was determined at 24, 48 and 72 hr as described in Methods. The four type 2 mAbs shown are 1-9 (-■-); 2-6 (-○-); 3-5 (-◇-) and 3-9 (-△-). Type 3 mAb is 2-13 (-◆-); control uptake (-□-). Each point is the mean of duplicate samples; the vertical bars represent the range.

of Cbl. For these experiments we used a physiologic concentration of Cbl (370 pM) to study the effect of the Cbl-blocking mAb on the cellular uptake of Cbl. We also observed substantial blocking of the uptake of Cbl even when the concentration of Cbl in the culture medium (880 pM) exceeded that found in normal plasma (150–600 pM). Carmel and Linker-Israeli (15) have previously reported the generation of two different mAbs against TCII. Neither mAb inhibited the binding of Cbl to TCII but one of the two inhibited by 60% the uptake of Cbl by K562 cells.

Cbl deficiency in humans perturbs a number of essential metabolic pathways. The consequent

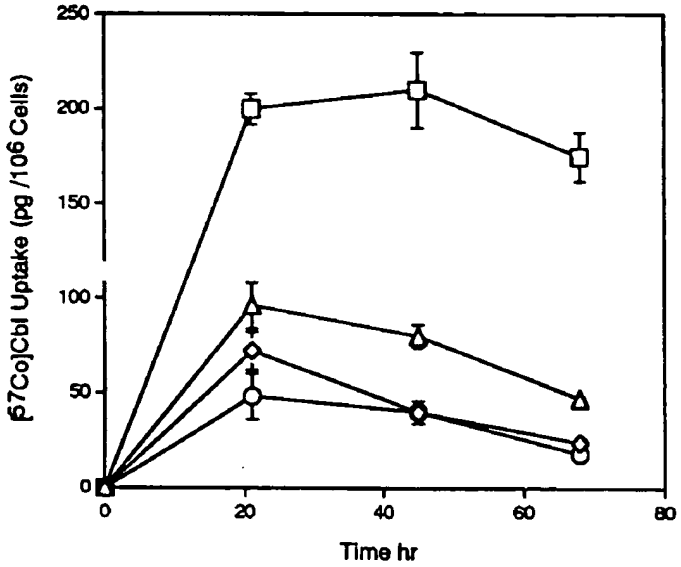


FIG. 5. The effect of type 1 (receptor blocking) mAb on the uptake of TCII-Cbl by K562 cells in culture. The mAb was incubated with TCII-[^{57}Co]Cbl overnight and the mixture was added to the cells in culture. The uptake of [^{57}Co]Cbl by the cells was determined at 24, 48 and 72 h as described in Methods. The three type 1 mAbs are 2-2 (-△-); 3-11 (-○-) and 4-7 (-◇-); control uptake, (-■-). Each point is the mean of triplicate samples; the vertical bars represent the range.

deficiency of methyl-Cbl impairs the methylation of homocysteine resulting in a decrease in methionine and its important metabolite, S-adenosylmethionine (16). A second consequence of methyl-Cbl deficiency is the trapping of N^5-methyltetrahydrofolate, the source of the carbon unit for methylation of homocysteine, and this also reduces the pool of folate cofactors that are essential for nucleic acid synthesis (6). The hematologic sequela of this deficiency is megaloblastic hematopoiesis with intramedullary cell death that is apparently due to apoptosis (17). For this reason, perturbation of Cbl metabolism has been sought as a means to control proliferation of neoplastic cells.

The mAb(s) we generated against TCII may be an alternative to anti-metabolites that target an intracellular pathway(s) and that have secondary and often deleterious effects. In preliminary studies on the biological effect of blocking Cbl uptake (18), we have also observed that K562 cells cultured for 12–15 days with type 1 mAb induces morphological characteristics of apoptotic cell death thus duplicating an effect of folate deficiency (17).

A clinical observation that has never been satisfactorily clarified is that patients with severe Cbl deficiency who have folate trapped as N^5methyltetrahydrofolate may have a hematologic response to daily administration of folic acid in excess of 400 μg (19). The induction of isolated Cbl depletion of sufficient magnitude to cause apoptosis in cultured cells by blocking the uptake of TCII-Cbl could provide the experimental model to precisely quantify the level of folate required to bypass this "methyl trap" and provide sufficient folate cofactors to restore nucleic acid synthesis. In addition, the inhibitory effect of the mAbs on the uptake of TCII-Cbl by neuronal cells and especially, the Schwann cells that deposit myelin around neuronal axons, may provide the biochemical basis for the demyelinization that occurs in Cbl deficiency and rarely seen in folate deficiency.

ACKNOWLEDGMENTS

This work was supported by Grant R01 DK28561 from the National Institutes of Health and a Merit Review award from the Veterans Affairs.

REFERENCES

1. Rothenberg, S. P., Weiss, J. P., and Cotter, R. (1970) *Brit. J. Haematol.* **40,** 401–414.
2. Quadros, E. V., Rothenberg, S. P., and Jaffe, E. A. (1989) *Amer. J. Physiol.* **256,** 296–303.
3. Donaldson, R. M., Brand, M. M., and Serfilippe, D. (1977) *N. Engl. J. Med.* **296,** 1427–1430.
4. Flavin, M., and Ochoa, S. (1957) *J. Biol. Chem.* **229,** 965–979.
5. Brot, N., Taylor, R., and Weisbach, H. (1966) *Arch. Biochem. Biophys.* **114,** 256–259.
6. Herbert, V., and Zaluski, R. (1962) *J. Clin. Invest.* **41,** 1263–1271.
7. Stabler, S. P., Allen, R. H., Savage, D. G., and Lindenbaum, J. (1990) *Blood* **76,** 871–881.
8. Perlman, D., Perlman, K. L., Williams, T. H., Schomer, U., and Izumi, Y. (1979) *in* Vitamin B12 (Zagalak, B., and Freidrich, W., Eds.), pp. 609–624, de Grayter, Berlin, Germany.
9. Deakon, R., Perry, J., Lumb, M., Chanarin, I., Minty, B., Halsey, M. J., and Nunn, J. F. (1978) *Lancet* **2,** 1023–1024.
10. Quadros, E. V., Jackson, B., Hoffbrand, A. V., and Linnell, J. C. (1979) *in* Vitamin B12 and Intrinsic Factor (Zagalak, B., and Friedrich, W., Eds), pp. 1045–1054, de Gruyter, Berlin, Germany.
11. Amess, J. A. L., Burman, J. F., Rees, G. M., Nancekievill, D. G., and Mollin, D. L. (1978) *Lancet* **2,** 339–342.
12. Quadros, E. V., Rothenberg, S. P., Pan, Y-C. E., and Stein, S. (1986) *J. Biol. Chem.* **261,** 15455–15460.
13. Allen, R. H., and Majerus, P. W. (1972) *J. Biol. Chem.* **247,** 7709–7717.
14. Gottlieb, C., Lan, K-S., Wasserman, L. R., and Herbert, V. (1965) *Blood* **25,** 875–884.
15. Carmel, R., and Linker-Israeli, M. (1988) *Proc. Soc. Exptl. and Biol. Med.* **188,** 77–81.
16. Weir, D. G., Molloy, A., Keating, J. N., McPartlin, J., Kennedy, S., Blancheflower, J., Rice, D., and Scott, J. M. (1990) *in* Biomedicine and Physiology of Vitamin B12 (Linnell, J. C., and Bhatt, H. R., Eds), pp. 129–151. Children's Medical Charity, London, UK.
17. Koury, M. J., and Horne, D. W. (1994) *Proc. Nat. Acad Sci.* **91,** 4067–4071.
18. Quadros, E. V., McLoughlin, P., Rothenberg, S. P., Morgan, A. C., Shikorska-Walker, M., and Walker, R. (1995) *Blood* **86,** 125a.
19. Marshall, R. A., and Jandl, J. H. (1960) *Arch. Int. Med.* **105,** 352–360.

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	~~ASAP~~	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	~~Part~~ Pathare et al
Journal/Book Title:	Bioconjugate Chemistry NPL
Article Title:	
Volume (Issue):	8(2)
Pages:	161-172
Year of Publication:	1997
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

Synthesis of Cobalamin Dimers Using Isophthalate Cross-Linking of Corrin Ring Carboxylates and Evaluation of Their Binding to Transcobalamin II

Pradip M. Pathare,[†] D. Scott Wilbur,*[,†] Donald K. Hamlin,[†] Shannon Heusser,[†] Edward V. Quadros,[‡] Patricia McLoughlin,[‡] and A. Charles Morgan[†,§]

Department of Radiation Oncology, University of Washington, Seattle, Washington 98195, VA Medical Center–SUNY Health Science Center, Brooklyn, New York 11209, and Receptagen Corporation, Edmonds, Washington 98020. Received August 21, 1996[⊗]

Several cobalamin (Cbl) dimers have been prepared for evaluation as potential antiproliferative agents in the treatment of AIDS-related lymphoma. The Cbl dimers were synthesized by cross-linking Cbl carboxylates, produced by acid hydrolysis of the *b*-, *d*-, and *e*-propionamide side chains of cyanocobalamin (CN-Cbl), through an isophthalate molecule. Linking molecules were used between the Cbl carboxylates and the isophthalate moiety. The linkers were incorporated to provide a distance between the two Cbl molecules such that the dimeric Cbls might bind two molecules of transcobalamin II (TCII), the Cbl transport protein in plasma. Initially, the linking moiety used was 1,12-diaminododecane, but the resulting dimers had low aqueous solubility. To improve the solubility of the dimers, 4,7,10-trioxa-1,13-tridecanediamine was employed as the linking moiety. This improved the water solubility of the dimers considerably, while retaining the distance between the Cbl molecules at 41–42 Å (fully extended). To introduce additional substitution on Cbl dimers, 5-aminoisophthalic acid was used as the cross-linking reagent. *p*-Iodobenzoyl and *p*-(tri-*n*-butylstannyl)benzoyl conjugates of 5-aminoisophthalate were synthesized and used to prepare Cbl dimers. The stannylbenzoyl-conjugated Cbl dimers were prepared as precursors to be used in radioiodination reactions, and the iodobenzoyl-conjugated Cbl dimers were prepared as HPLC standards for the radioiodinated product. Attempts to iodinate/radioiodinate the stannylbenzoyl Cbl dimers were unsuccessful. Although an explanation for this is not readily apparent, the failure to react may be due to the lipophilicity of the linker used and the steric environment of the two Cbl moieties. A biotinylated derivative of 5-aminoisophthalate was also synthesized and used to prepare biotinylated-Cbl dimers. In a competitive rhTCII binding assay with [^{57}Co]CN-Cbl, Cbl dimers containing the lipophilic diaminododecane linking moiety had decreased binding avidities compared to those of Cbl monomers substituted at the same corrin ring carboxylate. However, Cbl dimers containing the water-solubilizing trioxadiamine linker appeared to have avidities similar to those of the Cbl monomers.

INTRODUCTION

Cobalamins (Cbls[1]) are cofactors in enzymatic pathways associated with DNA and protein synthesis in cells (*1*, *2*). Actively dividing cells, such as hematopoietic cells in bone marrow, avidly take up Cbls. Importantly, it has been noted that Cbls play a significant metabolic role in human leukemia cells (*3*, *4*). These facts have led us to investigate the application of Cbl depletion to the therapy of rapidly dividing and proliferating cells found in leukemias and lymphomas. Evidence for such an antiproliferative therapeutic approach is not without precedent as previous investigations have shown that therapeutic responses could be obtained in patients when they were isolated in an atmosphere containing the anesthetic agent nitrous oxide (N_2O) (*5*, *6*). N_2O administration causes an oxidative inactivation of Cbl, depleting cells in the body of metabolically active forms of Cbl. The use of N_2O, while effective for inactivation of Cbl, presents several problems for its general application to the therapy of leukemias or lymphomas. One major problem is that patients are required to stay in an atmosphere of N_2O for extended periods of time, which results in extended hospitalization and the need for constant patient monitoring. Additionally, serious neurological side effects have been observed in some patients undergoing this treatment. Further, the nonspecific nature of the oxidation of Cbl with N_2O can have effects on many different biological systems and may lead to a number of toxicities. Thus, an alternative approach for depletion of cellular Cbl was conceived.

We have begun to investigate a novel approach to depletion of cellular Cbl which is directed at developing a pharmaceutical capable of blocking entry of endogenous Cbls into cells. Since the entry of Cbls into cells at physiological concentrations appears to be completely dependent on a receptor-mediated process (*7*), we are investigating synthetic Cbl derivatives designed to in-

* Address correspondence to this author at the Department of Radiation Oncology, University of Washington, 2121 N. 35th St., Seattle, WA 98103-9103 [telephone (206) 685-3085; fax (206) 685-9630; e-mail dswilbur@u.washington.edu].

[†] University of Washington.

[‡] VA Medical Center–SUNY Health Science Center.

[§] Receptagen Corp.

[⊗] Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: Cbl(s), cobalamin(s); CN-Cbl, cyanocobalamin; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; EDC, 1-[3-(dimethylamino)-propyl]-3-ethylcarbodiimide; 2HEDS, 2-hydroxyethyl sulfide; HSA, human serum albumin; LC, liquid chromatography; 3NBA, 3-nitrobenzyl alcohol; NCS, *N*-chlorosuccinimide; NHS, *N*-hydroxysuccinimide; PBS, phosphate-buffered saline; rt, room temperature; TCII, transcobalamin II; rhTCII, recombinant human transcobalamin II; TFP, tetrafluorophenyl; TFP-OH, tetrafluorophenol; TFP-OTFA, tetrafluorophenyl trifluoroacetate.

S1043-1802(97)00003-7 CCC: $14.00

terfere with the receptor-mediated cellular uptake of endogenous Cbls. It is known that cellular uptake of Cbls is a complex and highly regulated process which involves (1) transport of Cbl in plasma via a high-affinity serum Cbl binding protein, transcobalamin II (TCII) (*8*); (2) cell surface binding of the Cbl/TCII complex with a glycoprotein receptor (*9*); (3) internalization of the Cbl/TCII/receptor complex; and (4) release of the Cbl to the cytoplasm. Importantly, it has been shown in leukemia cells (e.g. L1210 and K562 cell lines) that the Cbl/TCII cell surface receptor density, which is directly related to Cbl uptake, is up-regulated during proliferation and down-regulated during quiescence (*10*). Although the process of receptor-mediated uptake and delivery of Cbls to cytoplasm is not fully understood, it has been shown that the lysosomotropic agent chloroquine and the monocarboxylate proton ionophore monensin can drastically decrease the Cbl/TCII surface receptor concentration in K562 leukemia cells (*10*). The lack of information available on the effect of alterations in the Cbl molecule on receptor trafficking and release of Cbl to cytoplasm makes it impossible to have a rational design of Cbl derivatives. Therefore, our initial investigations have involved synthesizing a number of Cbl derivatives containing a variety of appended groups for evaluation in cellular proliferation assays. To focus our synthetic efforts somewhat, we reasoned that Cbl derivatives containing appended highly lipophilic groups (e.g. fatty acids), highly ionic groups (e.g. alkyl sulfonates), or lysosomotropic agents (e.g. polyamines) (*11*) might result in retention of the Cbl, and possibly the retention of TCII/cell surface receptor, in endosomes and/or lysosomes.

When considering possible Cbl derivatives for synthesis, we became intrigued by derivatives that contain two or more Cbl moieties on the same molecule. Of the many derivatives that might be prepared, it appeared that binding with more than one TCII molecule had a higher potential for altering the receptor recycling process. Following this logic, we became interested in dimeric Cbl derivatives as they presented less of a synthetic challenge than molecules with multiple Cbl moieties. Since our approach was to utilize the Cbl/TCII receptor-mediated cell internalization process, it was apparent that any synthetic Cbl derivative prepared must bind with the plasma protein TCII as effectively as endogenous Cbl. Previous studies by this group (*12, 13*) and several other groups (*14–17*) have shown that conjugation of chemical moieties on the corrin ring side chains provides a stable attachment to the Cbl moiety which, depending on the location of attachment, has a varying effect on the binding with TCII. That variability of binding has been found to range from 3 orders of magnitude decreased binding relative to cyanocobalamin (CN-Cbl), **1**, for conjugates at the *c*-acetamide side chain, to nearly equivalent binding to TCII for the *e*-propionamide side chain conjugates (*12*). Thus, cobalamin dimers that were linked through corrin ring attachments were targeted. As with other Cbl derivatives, we felt that the cobalamin dimers should be designed such that it was possible to append functional groups that could change the physical nature (e.g. lipophilicity and ionic nature), or add lysosomotropic properties, to the dimers, so a trifunctional cross-linking moiety was included in the design. Further, it seemed important to design the Cbl dimers in a manner that separated the Cbl moieties by a significant distance such that binding with two TCII molecules might be achieved.

Reported herein are the synthesis and preliminary binding studies of 14 Cbl dimers. The dimers prepared employ isophthalic acid or aminoisopththalic acid moieties to cross-link corrin ring Cbl-carboxylates (*b*-, *d*-, or *e*-isomers). Cross-linking was accomplished by incorporating linker molecules, containing two terminal amino groups, between the Cbl carboxylates and the carboxylates of isophthalic acid. As part of the investigation, Cbl dimers containing arylstannanes were prepared such that radioiodine might be incorporated through an iododestannylation reaction (*18, 19*). Biotinylated Cbl dimers were also prepared in the investigation. Binding of 11 Cbl dimers with recombinant human TCII (rhTCII), relative to [^{57}Co]CN-Cbl, was measured in a competitive binding assay.

EXPERIMENTAL PROCEDURES

General. All chemicals purchased from commercial sources were of analytical grade or better and were used without further purification. Cyanocobalamin (CN-Cbl; vitamin B_{12}) was obtained from Sigma Chemical Co. (St. Louis, MO). *N*-Hydroxysuccinimide and isophthaloyl dichloride were purchased from Lancaster Synthesis Inc. (Windham, NH). All other reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI). Solvents for HPLC analysis were obtained as HPLC grade and were filtered (0.2 μm) prior to use. Ion exchange chromatography was conducted with 200–400 mesh strongly basic anion, 2% cross-linked Dowex 1 chloride (Aldrich). Amberlite XAD-2 nonionic polymeric adsorbent and octadecyl functionalized silica gel for column chromatography were also obtained from Aldrich. Bio-Sil NH_2 (aminopropyl bonded silica) (40–63 μm) for column packing was purchased from Bio-Rad Laboratories (Hercules, CA). Phosphate-buffered saline (PBS) was prepared as a solution containing 8.1 mM Na_2PO_4, 1.2 mM KH_2PO_4, and 138 mM NaCl, pH 7.4. Human serum albumin (HSA) was obtained from Miles, Inc. (Elkhart, IN).

Molecular modeling of Cbl dimers was conducted to estimate the distance between the Cbl moieties. The modeling was conducted with ChemDraw Plus/Chem3D Pro software (CambridgeSoft Corp., Burlington, MA) on a Macintosh 8100/80 computer. Structures of the Cbl cross-linking reagents were drawn (fully extended) and minimized for structural error and energy, and interatomic distances were obtained directly from the computer program.

Spectroscopic Data. ^{1}H NMR spectra were obtained on either a Bruker AC-300 (300 MHz) or a Bruker AC-500 (500 MHz) instrument. The chemical shifts are expressed as parts per million using tetramethylsilane as an internal standard (δ = 0.0 ppm). IR data were obtained on a Perkin-Elmer 1420 infrared spectrophotometer. UV data were obtained on a Perkin-Elmer Lambda 2 UV–vis spectrophotometer or a Shimadzu UV 160U spectrophotometer. UV absorbances were obtained as previously described (*12*). Mass spectral data were obtained on a VG 70SEQ mass spectrometer with 11250J data system. Fast atom bombardment (FAB^+) mass spectral data were obtained at 8 kV using a matrix of 3-nitrobenzyl alcohol (3NBA) or a matrix of 90% thioglycerol, 9% DMSO, and 1% TFA (DMIX). We were unable to find conditions for obtaining mass spectral data with the stannylbenzoyl-Cbl dimers **27–29**.

Identity of the Cbl derivatives was established by mass spectral and NMR data (see Supporting Information). Elemental analyses were not obtained for the Cbl dimers due to the difficulties encountered previously with monomeric Cbls (*12, 13*). Purity of the Cbl derivatives was established by HPLC analysis (see Supporting Information).

Analytical Chromatography. HPLC separations of compounds were obtained on a Hewlett-Packard quater-

nary 1050 gradient pumping system with a variable wavelength UV detector (360 nm). Analysis of the HPLC data was conducted on Hewlett-Packard HPLC Chemstation software. All reactions were monitored by HPLC.

Separations of the CN-Cbl, **1**, and Cbl derivatives **2**–**10** were conducted on an aminopropyl-silica column at a flow rate of 1 mL/min. The HPLC separations of the precursor compounds **1**–**7** were conducted on a 5 μm, 4.6 mm × 250 mm aminopropyl column (Rainin microsorb-MV amino column) eluting with 58 mM pyridine acetate, pH 4.4, in H_2O/THF (96:4) solution (*9*). Retention times for the Cbls evaluated with this system were as follows: **1** = 2.7 min; **2** = 3.8 min; **3** = 4.4 min; **4** = 5.1 min; **5** = 2.2 min; **6** = 2.2 min; **7** = 2.2 min; **8** = 2.3 min; and **10** = 2.3 min.

HPLC separations for benzoylaminoisophthalate **11**–**14** were conducted on a Hewlett-Packard LiChrospher 100 RP-18 (5 μm; 4.6 mm × 125 mm) C_{18} column using a gradient solvent system. Solvent A in the gradient was MeOH. Solvent B was H_2O. Starting from 70% A, the initial solvent mixture was held for 2 min, then the gradient was increased to 100% A over the next 10 min, and 100% A was held for 5 min. The gradient was decreased in percentage of A to 70% over the next 5 min. Retention times under these conditions were as follows: **11** = 3 min (solvent front); **12** = 6.6 min; **13** = 14.8 min; and **14** = 21.9 min.

HPLC separations for aminocaproate–biotin derivatives **15**–**20** and for dimers **21**–**31** were conducted on a C_{18} reversed phase column employing a gradient. Solvent A in the gradient was methanol. Solvent B was aqueous 1% acetic acid. The gradient was begun at 40% A and was held at that composition for 2 min, and then the percentage of A was linearly increased to 100% over the next 10 min. The gradient was held at 100% A for 20 min. Retention times under these conditions for dimers were as follows: **15** = 6.1 min; **16** = 12.9 min; **17** = 7.2 min; **18** = 12.7 min; **19** = 8.4 min; **20** = 14.8 min; **21** = 12.6 min; **22** = 12.3 min; **23** = 13.0 min; **24** = 13.5 min; **25** = 13.8 min; **26** = 13.9 min; **27** = 8.6 min; **28** = 8.7 min; **29** = 9.0 min; **30** = 12.8 min; and **31** = 12.8 min.

For HPLC chromatography of dimers **32**–**34**, reversed phase chromatography was conducted at a flow rate of 1 mL/min. Solvent A in the gradient was methanol. Solvent B was H_2O. The gradient was held at the starting mixture of 70% A for 2 min, and then the percentage of A was linearly increased to 100% over the next 10 min. Retention times for the compounds examined under these conditions were as follows: **32** = 10.4 min; **33** = 10.8 min; and **34** = 10.8 min.

Preparative LC. A preparative LC system containing a Rainin Rabbit-plus peristaltic pump and a Dynamax Model FC-1 fraction collector was used to obtain pure samples of Cbl derivatives. Compounds were separated on either an aminopropyl silica column (1000 mm × 25 mm; 40–63 μm; Alltech) or a C_{18} reversed phase column (25 mm × 500 mm; octadecyl; Aldrich). Isolation of purified products was aided by evaluation of collected fractions from the preparative LC on an analytical HPLC column.

Preparation of Cyanocobalamin Monocarboxylic Acids 2, 3, or 4. The *b*-, *d*-, and *e*-Cbl monocarboxylates (**2**, **3**, and **4**, respectively) were prepared as previously reported (*12*). Briefly, CN-Cbl was hydrolyzed in 0.1 N HCl over 10 days at room temperature. Following the hydrolysis reaction, the isomeric monocarboxylates were separated from starting CN-Cbl and from di- and triacids by ion exchange chromatography. Separation of the individual carboxylate isomers was accomplished by preparative liquid chromatography on an aminopropyl-silica column (25 mm × 1000 mm) at a flow rate of 0.15 mL/min.

General Procedure for Conjugation of 2, 3, or 4 with 1,12-Diaminododecane; Synthesis of 5, 6, and 7. The conjugation of the Cbl-monocarboxylates with diaminododecane was accomplished as previously described (*12*). Briefly, reaction of **2**, **3**, or **4** with diaminododecane, EDC, or NHS and in a 1:1 mixture of DMF and H_2O for 4 days yielded the desired compounds after purification by ion exchange chromatography.

Conjugation of Cyanocobalamin Monocarboxylic Acid with 4,7,10-Trioxa-1,13-Tridecanediamine; Synthesis of 8 and 10. A 2.0 g (1.47 mmol) quantity of a CN-Cbl monocarboxylic acid, **2** or **4**, and 0.68 g (5.9 mmol) of NHS were dissolved in 100 mL of water. To that mixture was added 1.46 g (29 mmol) of NaCN, then 16 g (36 mmol) of 4,7,10-trioxa-1,13-tridecanediamine was added, and the pH was adjusted to 6 with 1 N HCl. To that solution was added 1.14 g (5.9 mmol) of EDC, and the pH of the solution was readjusted to 5.5. The reaction mixture was then stirred overnight in the dark at rt. In five intervals of 6–14 h, 0.68 g of NHS and 1.14 g of EDC were added to the solution, with the pH value readjusted to 5.5 each time. After a total reaction time of 4 days, the solution was evaporated to dryness. The residue was washed with 100 mL of acetone, and the solvent was decanted. The remaining solid was dissolved in 50 mL of H_2O and applied to an Amberlite XAD-2 (200 g; 4 cm × 60 cm) column. The column was eluted with 1 L of water, and then the desired product was eluted with 500 mL of methanol. The methanol fractions were evaporated to dryness, and the residue was dissolved in 25 mL of water and was applied to a ion exchange column (100 g; 2.5 cm × 60 cm; acetate form; 200–400 mesh). The final product was eluted using 250 mL of water, thereby leaving nonconverted Cbl-acid bound to the column, which was later eluted with 0.04 mol/L sodium acetate buffer, pH 4.7. The fractions containing the final product were evaporated to dryness and then washed with acetone and filtered. The solid obtained was recrystallized from aqueous acetone.

b-Isomer (**8**): yield, 2.0 g (87%); mp, 213–217 °C with decomposition; ^{1}H NMR (MeOH-d_4) δ 0.44 (s, 3H), 1.17 (d, 5H), 1.25 (d, 4H), 1.36 (d, 7H), 1.45 (s, 4H), 1.74 (m, 10H), 1.88 (s, 11H), 2.27 (d, 8H), 2.34 (m, 11H), 2.56 (m, 11H), 3.17 (t, 3H), 3.2 (m, 9H), 3.3 (m, 6H), 3.4 (m, 4H), 3.5 (s, 7H), 3.58 (s, 8H), 3.6 (m, 11H), 3.7 (m, 1H), 3.88 (m, 1H), 4.07 (m, 1H), 4.1 (m, 1H), 4.17 (m, 1H), 4.3 (m, 1H), 4.5 (m, 1H), 4.6 (m, 1H), 6.04 (d, 1H), 6.27 (s, 1H), 6.52 (s, 1H), 7.13 (d, 1H), 7.25 (s, 1H); MS (FAB$^+$) mass calcd for $C_{73}H_{109}N_{15}O_{18}CoP$ 1557, found 1558 $(M + H)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (H_2O) λ_{361} (ϵ = 17 500).

e-Isomer (**10**): yield, 1.5 g (65%); mp, 112–116 °C with decomposition; ^{1}H NMR (MeOH-d_4) δ 0.44 (s, 3H), 1.18 (s, 3H), 1.25 (d, 5H), 1.37 (d, 8H), 1.45 (s, 4H), 1.74 (m, 10H), 1.88 (s, 11H), 2.28 (d, 7H), 2.3 (m, 15H), 2.56 (d, 11H), 3.17 (t, 3H), 3.2 (t, 4H), 3.3 (m, 11H), 3.4 (m, 4H), 3.5 (s, 7H), 3.58 (d, 3H), 3.6 (m, 5H), 3.7 (m, 1H), 4.0 (m, 1H), 4.1 (d, 1H), 4.19 (m, 1H), 4.3 (m, 1H), 4.5 (d, 1H), 4.6 (m, 1H), 6.05 (d, 1H), 6.27 (s, 1H), 6.57 (s, 1H), 7.1 (d, 1H), 7.25 (s, 1H), MS (FAB$^+$) mass calcd for $C_{73}H_{109}N_{15}O_{18}CoP$ 1557, found 1558 $(M + H)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (H_2O) λ_{361} (ϵ = 12 800).

***p*-Iodobenzoyl-5-aminoisophthalic Acid, 12.** A 5.0 g (28 mmol) quantity of 5-amino-isophthalic acid, **11**, was dissolved in 30 mL of 1 N NaOH and placed in an ice/water bath. To the cold solution was added 7.5 g (28

mmol) of 4-iodobenzoyl chloride in 60 mL of acetonitrile, dropwise. The thick white precipitate was stirred for 10 min before the ice/water bath was removed, and the mixture was allowed to stir for an additional 10 min. The reaction mixture was adjusted to pH 4 with acetic acid and the resulting solid collected. This solid was dissolved in 30 mL of 1 N NaOH and washed with 2 × 50 mL of ether. The resulting aqueous solution was filtered and acidified to pH 4 with acetic acid. The white precipitate was collected and dried under high vacuum to yield 11.6 g (99+%) of **12**: mp >300 °C; ^{1}H NMR (DMSO-d_6) δ 7.84 (d, 2H, J = 4.1 Hz), 7.94 (d, 2H, J = 4.2 Hz), 8.27 (s, 1H), 8.51 (d, 2H, J = 0.7 Hz); IR (Nujol, cm^{-1}) 3570, 3300, 1645, 1580, 1525, 760; HRMS (FAB$^+$; DMIX) (M + H)$^+$ mass calcd for $C_{15}H_{11}INO_5$ 411.9682, found 411.9696.

***p*-Iodobenzoyl-5-aminoisophthalate DiTFP Ester, 13.** A 5.0 g (12.2 mmol) quantity of **12** was suspended in 100 mL of anhydrous ethyl acetate. To this suspension was added 12.5 g (73 mmol) of 2,3,5,6-tetrafluorophenol (TFP-OH) followed by 5.0 g (24.2 mmol) of 1,3-dicyclohexylcarbodiimide (DCC). This suspension was stirred at rt for 3 days before filtering off the solid and washing with an additional 20 mL of ethyl acetate. The filtrate was evaporated to dryness. The resulting sticky white solid was suspended in 50 mL of acetonitrile, stirred for 30 min, and filtered to yield 3.75 g of **13** as a white solid (43%): mp 250–251 °C; ^{1}H NMR (DMSO-d_6) δ 7.81 (d, 2H, J = 4.3 Hz), 7.94 (d, 2H, J = 4.2 Hz), 8.04 (m, 2H), 8.57 (t, 1H, J = 1.4 Hz), 9.06 (d, 2H, J = 0.7 Hz); IR (Nujol, cm^{-1}) 3220, 3060, 1750, 1655, 1520, 1485, 1330, 1195, 1110, 1085, 955, 945; HRMS (FAB$^+$; 3NBA) (M + H)$^+$ mass calcd for $C_{27}H_{11}F_8INO_5$ 707.9554, found 707.9552.

***p*-(Tri-*n*-butylstannyl)benzoyl-5-aminoisophthalate DiTFP Ester, 14.** A 2.0 g (2.8 mmol) quantity of **13** was dissolved in 20 mL of dry toluene under argon. To this solution was added 2.8 mL (5.5 mmol) of bis-(tributyltin), followed by 40 mg (0.04 mmol) of tetrakis-(triphenylphosphine)palladium(0). The mixture was stirred at rt for 15 min before heating to 80 °C for 2 h. After 2 h, an additional 40 mg of palladium catalyst was added. Within 1 h the mixture had turned black. After cooling to rt, the toluene was removed by rotary evaporation. The resulting black oil was taken into 20 mL of ethyl acetate and dried onto 10 g of silica gel while on a rotary evaporator. The oil-coated silica was added to the top of a 250 g (40 × 3.5 cm) silica gel column. The column was initally eluted with hexanes containing 5% acetic acid, but after eluting with 600 mL, the solvent was changed to 90:10 hexanes/ethyl acetate (containing 5% acetic acid). Fractions 14–16 were combined and dried to yield 1.5 g of **14** as a white solid (62%): mp 120–123 °C; ^{1}HNMR ($CDCl_3$) δ 0.89 (t, 9H, J = 7.3 Hz), 1.11 (m, 6H), 1.36 (m, 15H), 1.55 (m, 6H), 7.07 (m, 2H), 7.62 (d, 2H, J = 4.1 Hz), 7.84 (d, 2H, J = 4.1 Hz), 8.38 (s, 1H), 8.76 (t, 1H, J = 1.6 Hz), 8.87 (d, 2H, J = 0.7 Hz); IR (Nujol, cm^{-1}) 1750, 1645, 1520, 1480, 1185, 1100, 1085; MS (FAB$^+$) mass calcd (isotopic abundance) 868 (38%), 869 (37%), 870 (75%), 871 (53%), 872 (100%), 873 (41%), 874 (21%); mass found 868 (47%), 869 (42%), 870 (82%), 871 (55%), 872 (100%), 873 (42%), 874 (26).

Biotin TFP Ester, 16. A 3.0 g (12.3 mmol) quantity of biotin, **15**, was dissolved in 60 mL of warm (70 °C) DMF under argon atmosphere. The solution was cooled to ambient temperature, and 2.79 g (13.5 mmol) of DCC was added, followed by 40.8 g (24.6 mmol) of TFP-OH. The reaction mixture was cooled to 0 °C and stirred at that temperature for 0.5 h. It was brought back to ambient temperature and stirred for another 4–5 h. The mixture was filtered and the filtrate evaporated to dryness. The resultant solid was washed with 50 mL of acetonitrile and dried to yield 5.0 g (98%) of **16** as a white solid: mp 185–187 °C (*20*); ^{1}H NMR (DMSO-d_6) δ 1.4 (m, 2H), 1.7 (m, 2H), 2.5 (t, 2H), 2.8 (t, 2H), 3.1 (m, 1H), 4.1 (m, 1H), 4.3 (m, 1H), 6.4 (d, 2H), 7.9 (m, 1H); IR (KBr, cm^{-1}) 3250, 2915, 1790, 1710, 1520, 1480, 1090.

Biotin–Aminocaproate TFP Ester, 18. A 0.99 g quantity (7.5 mmol) of 6-aminocaproic acid was dissolved in 75 mL of H_2O. To this mixture was added 0.5 mL of triethylamine, followed by a solution of 1.96 g (5 mmol) of **16** in warm acetonitrile (300 mL). The reaction was stirred overnight at rt. It was filtered, washed with 50 mL of H_2O, and dried under high vacuum to yield 0.870 g (47%) of **17**. Additional material was obtained by evaporating the filtrate to dryness, dissolving the residue in 75 mL of CH_3CN, and allowing the CH_3CN solution to cool to rt. The resultant solid was filtered, washed with warm acetonitrile, and dried under high vacuum to give an additional 0.6 g. Total yield of **17** was 1.47 g (79%): mp 225–227 °C; ^{1}H NMR (DMSO-d_6) δ 1.2–1.6 (m, 8H), 2.0 (t, 2H), 2.2 (t, 2H), 2.5 (dd, 2H), 2.8 (dd, 2H), 3.1 (m, 3H), 4.1 (m, 1H), 4.3 (m, 1H), 6.4 (d, 2H), 7.7 (m, 1H); IR (KBr, cm^{-1}) 3280, 2915, 1710, 1630, 1540, 1260, 1030.

A 1.0 g quantity (2.68 mmol) of **17** was dissolved in 50 mL of DMSO. To that solution was added 0.4 mL of triethylamine, followed by 1.05 g (4.02 mmol) of TFP-OTFA (*21*). The reaction mixture was stirred at rt for 15–20 min and then evaporated to dryness. The residue was washed with ether and dichloromethane. The resulting solid was dried under vacuum to yield 1.24 g (89%) of **18**: mp 139–141 °C; ^{1}H NMR (DMSO-d_6) δ 1.2 (t, 2H), 1.3–1.7 (m, 5H), 2.1 (t, 2H), 2.6 (dd, 2H), 2.8 (m, 4H), 3.1 (m, 4H), 4.2 (m, 1H), 4.4 (m, 1H), 6.4 (d, 2H), 7.8 (t, 1H), 8.0 (m, 1H); IR (KBr, cm^{-1}) 3300, 2940, 1785, 1690, 1640, 1520, 950; HRMS (FAB$^+$, 3NBA) (M + H)$^+$ mass calcd for $C_{22}H_{28}F_4N_3O_4S$ 506.1737, found 506.1732.

Biotin–Aminocaproate 5-Aminoisophthalic Acid DiTFP Ester, 20. A 0.35 g (0.67 mmol) quantity of **18** was dissolved in 40 mL of DMF. To that solution was added 80 μL of triethylamine, followed by 0.182 g (1.01 mmol) of 5-aminoisophthalic acid. The reaction was stirred at rt for 8 days, with triethylamine (80 μL) added every 24 h. It was then evaporated to dryness, and the residue was applied to a silica column. The column was initially eluted with 450 mL of acetonitrile, followed by 40 mL of methanol, and then DMF, collecting 20 mL fractions. The fractions containing the final product (HPLC monitored) were evaporated to dryness to yield 230 mg (65%) of **19**: mp 193–195 °C; ^{1}H NMR (DMSO-d_6) δ 1.3–1.7 (m, 8H), 2.1 (t, 2H), 2.3 (t, 2H), 2.6 (m, 2H), 2.8 (m, 2H), 3.1 (m, 3H), 4.1 (m, 1H), 4.3 (m, 1H), 6.4 (d, 2H), 7.8 (t, 1H), 8.1 (m, 1H), 8.46 (s, 2H); IR (KBr, cm^{-1}) 3280, 2920, 1710, 1690, 1640, 1240, 1100, 900; HRMS (FAB$^+$, 3NBA) (M + H)$^+$ mass calcd for $C_{24}H_{33}N_4O_7S$ 521.2070, found 521.2070.

A 200 mg (0.376 mmol) quantity of **19** was dissolved in 30 mL of DMF under argon atmosphere. To this solution was added 241 mg (0.94 mmol) of TFP-OTFA by a transfer using a double-ended needle. That addition was followed by addition of 112 μL of triethylamine. The reaction mixture was stirred at rt for 24 h (HPLC monitored) and then evaporated to dryness. The light brown oil was triturated with ether, and the solution was filtered and washed with 50 mL of additional ether to yield 250 mg (86%) of **20**: mp 135–137 °C; ^{1}H NMR (DMSO-d_6) δ 1.3–1.7 (m, 8H), 2.1 (t, 2H), 2.3 (t, 2H), 2.6 (m, 2H), 2.8 (m, 2H), 3.1 (m, 3H), 4.2 (m, 1H), 4.4 (m, 1H), 6.4 (d, 2H), 7.8 (t, 1H), 8.1 (m, 2H), 8.57 (s, 1H), 8.9 (s, 2H); IR (KBr, cm^{-1}) 3280, 2920, 1260, 1200, 1690,

1520, 1185, 950; HRMS $(M + H)^+$ mass calcd for $C_{36}H_{32}F_8N_4O_7S$ 817.1942, found 817.1920.

Conjugation of Cyanocobalamin Monocarboxylic Acid Diaminododecane with Isophthaloyl Dichloride; Synthesis of 21–23. To a solution of 0.300 g (0.192 mmol) of **5**, **6**, or **7** in 30 mL of DMF was added 18 μL of triethylamine. To that solution was added 0.195 g (0.096 mmol) of isophthaloyl dichloride over a period of 10–15 min. The reaction mixture was stirred at 55–60 °C for 48 h. It was then evaporated to dryness. The solid residue was dissolved in 20 mL of methanol/H_2O (7:3) and eluted on a preparative reversed phase column (500 mm × 25 mm) with the same solvent. The fractions containing the product were evaporated to dryness.

b-Acid Dimer (**21**): yield, 121 mg (38%); mp 220–222 °C with decomposition; ^{1}H NMR (D_2O) δ 0.43 (s, 6H, C-20 CH_3), 1.17 (s, 8H), 1.22 (d, 13H), 1.29 (s, 45H), 1.36 (d, 22H), 1.44 (s, 10H), 1.6 (m, 8H), 1.87 (s, 8H), 2.04 (m, 10H), 2.25 (s, 12H), 2.36 (m, 8H), 2.55 (d, 20H), 2.8 (m, 8H), 3.15 (m, 8H), 3.29 (s, 10H), 3.36 (m, 14H), 3.6 (m, 4H), 3.73 (m, 2H), 3.9 (d, 2H), 4.07 (m, 2H), 4.12 (m, 2H), 4.16 (m, 2H), 4.3 (m, 2H), 4.5 (m, 2H), 4.6 (s, 2H), 4.66 (m, 2H), 6.0 (s, 2H), 6.26 (d, 2H), 6.6 (s, 2H), 7.1 (s, 2H), 7.25 (s, 2H), 7.54 (t, 1H), 7.93 (d, 2H), 8.25 (s, 1H); MS (FAB^+) mass calcd for $C_{158}H_{228}N_{30}O_{30}Co_2P_2$ 3208,[2] found 3208 $(M)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) λ_{360} (ϵ = 33 900).

d-Acid Dimer (**22**): yield, 96 mg (30%); mp 217–220 °C with decomposition; ^{1}H NMR (D_2O) δ 0.43 (s, 6H, C-20 CH_3), 1.18 (s, 8H), 1.3 (m, 36H), 1.37 (m, 12H), 1.46 (s, 10H), 1.6 (m, 8H), 1.9 (d, 12H), 2.05 (m, 10H), 2.2 (d, 16H), 2.35 (m, 8H), 2.6 (d, 18H), 2.8-3.0 (m, 16H), 3.15 (m, 6H), 3.3 (s, 8H), 3.37 (m, 14H), 3.6 (m, 4H), 3.76 (m, 2H), 3.9 (d, 2H), 4.07 (m, 2H), 4.12 (m, 2H), 4.18 (m, 2H), 4.3 (m, 2H), 4.5 (m, 2H), 4.6 (s, 2H), 4.68 (m, 2H), 6.0 (s, 2H), 6.26 (d, 2H), 6.6 (s, 2H), 7.1 (s, 2H), 7.25 (s, 2H), 7.54 (t, 1H), 7.95 (d, 2H), 8.25 (s, 1H); MS (FAB^+) mass calcd for $C_{158}H_{228}N_{30}O_{30}Co_2P_2$ 3208, found 3208 $(M)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) λ_{360} (ϵ = 42 400).

e-Acid Dimer (**23**): yield, 96 mg (30%); mp 225–228 °C with decomposition; ^{1}H NMR (D_2O) δ 0.43 (s, 6H), 1.16 (s, 8H), 1.29 (m, 36H), 1.35 (d, 12H), 1.44 (s, 10H), 1.53 (m, 6H), 1.6 (m, 8H), 1.85 (s, 12H), 2.03 (m, 8H), 2.25 (d, 12H), 2.33 (m, 8H), 2.54 (d, 20H), 2.8 (m, 8H), 3.13 (m, 8H), 3.28 (s, 12H), 3.35 (m, 12H), 3.6 (m, 4H), 3.73 (m, 2H), 3.9 (d, 2H), 4.07 (m, 2H), 4.12 (m, 2H), 4.16 (m, 2H), 4.3 (m, 2H), 4.5 (m, 2H), 4.64 (m, 2H), 4.7 (s, 2H), 6.0 (s, 2H), 6.26 (d, 2H), 6.6 (s, 2H), 7.1 (s, 2H), 7.25 (s, 2H), 7.54 (t, 1H), 7.93 (d, 2H), 8.25 (s, 1H); MS (FAB^+) mass calcd for $C_{158}H_{228}N_{30}O_{30}Co_2P_2$ 3208, found 3209 $(M + H)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) λ_{360} (ϵ = 31 700).

Conjugation of Cyanocobalamin Monocarboxylic Acid Diaminododecane with *p*-Iodobenzoyl Aminoisophthalate; Synthesis of 24–26. To a solution containing 0.30 g (0.192 mmol) of **5**, **6**, or **7** in 40 mL of a 3:1 mixture of DMF/H_2O was added 18 μL of triethylamine. To that solution was added 68 mg (0.096 mmol) of **13** over a 5–10 min period. The reaction mixture was stirred at rt for 4–5 h and then evaporated to dryness. The solid residue was dissolved in 20 mL of a 4:1 MeOH/H_2O mixture and applied to a preparative reversed phase column (500 mm × 25 mm), which was eluted with the same solvent. The fractions containing the product were evaporated to dryness.

b-Acid Dimer (**24**): yield, 258 mg (70%); mp 285–290 °C with decomposition; ^{1}H NMR (D_2O) δ 0.43 (s, 6H), 1.17 (s, 8H), 1.22 (d, 13H), 1.29 (s, 45H), 1.36 (d, 22H), 1.44 (s, 10H), 1.6 (m, 8H), 1.86 (s, 12H), 2.04 (m, 10H), 2.25 (s, 12H), 2.36 (m, 8H), 2.55 (d, 20H), 2.83 (m, 8H), 3.15 (m, 8H), 3.29 (s, 10H), 3.36 (m, 8H), 3.58 (m, 2H), 3.65 (m, 2H), 3.75 (m, 2H), 3.9 (d, 2H), 4.06 (m, 2H), 4.12 (m, 2H), 4.16 (m, 2H), 4.3 (m, 2H), 4.5 (m, 2H), 4.57 (s, 2H), 4.65 (m, 2H), 6.0 (s, 2H), 6.26 (d, 2H), 6.5 (s, 2H), 7.1 (s, 2H), 7.25 (s, 2H), 7.7 (d, 2H), 7.89 (d, 2H), 7.98 (s, 1H), 8.26 (s, 2H); MS (FAB^+) mass calcd for $C_{165}H_{232}O_{31}N_{31}Co_2P_2I$ 3453, found 3453 $(M)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) λ_{360} (ϵ = 41 500).

d-Acid Dimer (**25**): yield, 280 mg (76%); mp 230–233 °C with decomposition; ^{1}H NMR (D_2O) δ 0.43 (s, 6H), 1.19 (s, 8H), 1.3 (m, 36H), 1.37 (d, 12H), 1.46 (s, 10H), 1.63 (m, 8H), 1.87 (s, 12H), 2.05 (m, 10H), 2.27 (d, 16H), 2.35 (m, 8H), 2.6 (d, 18H), 2.8 (s, 8H), 3.0 (s, 10H), 3.15 (m, 8H), 3.3 (d, 8H), 3.37 (m, 14H), 3.6 (m, 2H), 3.68 (d, 2H), 3.76 (m, 2H), 3.9 (d, 2H), 4.07 (m, 2H), 4.12 (m, 2H), 4.18 (m, 2H), 4.3 (m, 2H), 4.5 (m, 2H), 4.64 (m, 4H), 6.0 (s, 2H), 6.26 (d, 2H), 6.6 (s, 2H), 7.1 (s, 2H), 7.25 (s, 2H), 7.7 (d, 2H), 7.9 (d, 2H), 7.99 (d, 1H), 8.28 (s, 2H); MS (FAB^+) mass calcd for $C_{165}H_{232}O_{31}N_{31}Co_2P_2I$ 3453, found 3453 $(M)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) $\lambda_{360.6}$ (ϵ = 48 900).

e-Acid Dimer (**26**): yield, 265 mg (72%); mp 253–255 °C with decomposition; ^{1}H NMR (D_2O) δ 0.43 (s, 6H), 1.16 (s, 8H), 1.22 (d, 12H), 1.33 (m, 36H), 1.43 (s, 10H), 1.53 (m, 6H), 1.6 (m, 8H), 1.86 (s, 12H), 2.03 (m, 8H), 2.25 (d, 12H), 2.33 (m, 8H), 2.54 (d, 20H), 2.8 (s, 4H), 3.0 (s, 4H), 3.28 (s, 10H), 3.35 (m, 8H), 3.58 (m, 2H), 3.65 (m, 2H), 3.73 (m, 2H), 3.88 (d, 2H), 4.05 (m, 2H), 4.1 (m, 2H), 4.17 (m, 2H), 4.3 (m, 2H), 4.5 (m, 2H), 4.57 (s, 2H), 4.63 (m, 2H), 6.0 (s, 2H), 6.26 (d, 2H), 6.5 (s, 2H), 7.1 (s, 2H), 7.25 (s, 2H), 7.7 (d, 2H), 7.89 (d, 2H), 7.98 (s, 1H), 8.26 (s, 2H); MS (FAB^+) mass calcd for $C_{165}H_{232}O_{31}N_{31}Co_2P_2I$ 3453, found 3452 $(M)^2$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) λ_{360} (ϵ = 48 200).

Conjugation of Cyanocobalamin Monocarboxylic Acid Diaminododecane with *p*-(Tri-*n*-butylstannyl)-benzoyl Aminoisophthalate; Synthesis of 27–29. To a solution containing 0.10 g (0.065 mmol) of **5**, **6**, or **7** in 40 mL of a 3:1 mixture of DMF/H_2O was added 6 μL of triethylamine. To that solution was added 28 mg (0.033 mmol) of **14** over a 5–10 min period. The reaction mixture was stirred at rt for 12–14 h and then evaporated to dryness. The residue was washed with 100 mL of acetone, and the solvent was decanted.

b-Acid Dimer (**27**): yield, 93 mg (72%); mp > 300 °C; ^{1}H NMR (D_2O) δ 0.43 (s, 6H, C-20 CH_3), 0.88 (t, 9H), 1.12 (t, 12H), 1.17 (d, 8H), 1.22 (d, 13H), 1.29 (s, 45H), 1.36 (d, 22H), 1.44 (s, 10H), 1.6 (m, 8H), 1.87 (d, 12H), 2.04 (m, 10H), 2.25 (s, 12H), 2.36 (m, 8H), 2.55 (d, 20H), 2.8 (m, 8H), 3.15 (m, 8H), 3.29 (s, 10H), 3.36 (m, 14H), 3.6 (m, 4H), 3.73 (m, 2H), 3.9 (d, 2H), 4.07 (m, 2H), 4.12 (m, 2H), 4.16 (m, 2H), 4.3 (m, 2H), 4.5 (m, 2H), 4.66 (m, 2H), 6.0 (s, 2H), 6.26 (d, 2H), 6.6 (s, 2H), 7.1 (s, 2H), 7.25 (s, 2H), 7.6 (d, 2H), 7.9 (d, 2H), 7.98 (br s, 1H), 8.28 (br s, 2H); IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) λ_{360} (ϵ = 59 300)

d-Acid Dimer (**28**): yield, 90 mg (70%); mp 208–212 °C with decomposition; ^{1}H NMR (D_2O) δ 0.43 (s, 6H), 0.88 (t, 9H), 1.15 (t, 12H), 1.19 (s, 8H), 1.3 (m, 36H), 1.37 (d, 12H), 1.46 (s, 10H), 1.6 (m, 8H), 1.9 (s, 12H), 2.05 (m, 10H), 2.28 (d, 16H), 2.35 (m, 8H), 2.6 (d, 18H), 2.8–2.9

[2] Obtaining exact mass values for molecules of >3000 amu is difficult. It is believed that the difference of 1 mass unit obtained is a reflection of the difficulty in assigning mass units to the spectrometer data, not an indication that the mass is different from that calculated for the desired compound.

(m, 16H), 3.15 (m, 8H), 3.3 (s, 8H), 3.37 (m, 14H), 3.6 (m, 4H), 3.76 (m, 2H), 3.9 (d, 2H), 4.07 (m, 2H), 4.12 (m, 2H), 4.18 (m, 2H), 4.3 (m, 2H), 4.5 (m, 2H), 4.68 (m, 2H), 6.0 (s, 2H), 6.26 (d, 2H), 6.6 (s, 2H), 7.1 (s, 2H), 7.25 (d, 2H), 7.6 (d, 2H), 7.9 (d, 2H), 7.99 (br s, 1H), 8.28 (br s, 2H); IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) λ_{360} (ϵ = 47 700).

e-Acid Dimer (**29**): yield, 100 mg (78%); mp 202–205 °C with decomposition; ^{1}H NMR (D_2O) δ 0.43 (s, 6H), 0.88 (t, 9H), 1.12 (t, 12H), 1.15 (s, 8H), 1.29 (m, 36H), 1.35 (d, 12H), 1.44 (s, 10H), 1.53 (m, 6H), 1.6 (m, 8H), 1.86 (d, 12H), 2.03 (m, 8H), 2.25 (d, 12H), 2.33 (m, 8H), 2.54 (d, 20H), 2.8 (m, 8H), 3.13 (m, 8H), 3.28 (s, 10H), 3.35 (m, 10H), 3.6 (m, 4H), 3.73 (m, 2H), 3.9 (d, 2H), 4.05 (m, 2H), 4.1 (m, 2H), 4.17 (m, 2H), 4.3 (m, 2H), 4.5 (m, 2H), 4.6 (m, 2H), 6.0 (s, 2H), 6.26 (d, 2H), 6.6 (s, 2H), 7.1 (s, 2H), 7.25 (s, 2H), 7.6 (d, 2H), 7.9 (d, 2H), 7.98 (br s, 1H), 8.28 (br s, 2H); IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) λ_{360} (ϵ = 41 900).

Conjugation of Cyanocobalamin Monocarboxylic Acid Diaminododecane with Biotin–Aminocaproate Aminoisophthalate; Synthesis of 30 and 31. To a solution of containing 0.20 g (0.13 mmol) of **5**, **6**, or **7** in 40 mL of a 3:1 mixture of DMF/H_2O was added 12 μL of triethylamine. To that solution was added 50 mg (0.065 mmol) of **20** over a 5–10 min period. The reaction mixture was stirred at rt for 3 h and then was evaporated to dryness. The residue was washed with 100 mL of acetone, and the solvent was decanted.

b-Acid Dimer (**30**): yield, 124 mg (62%); mp 195–198 °C with decomposition; ^{1}H NMR (MeOH-d_4) δ 0.43 (s, 6H), 1.17 (s, 8H), 1.3 (m, 20H), 1.35 (m, 22H), 1.46 (s, 10H), 1.6 (m, 8H), 1.8 (m, 6H), 1.87 (m, 10H), 2.0 (m, 4H), 2.17 (s, 10H), 2.25 (s, 12H), 2.57 (d, 16H), 3.16 (m, 9H), 3.29 (s, 8H), 3.37 (m, 6H), 3.6 (m, 4H), 3.76 (m, 2H), 3.9 (d, 2H), 4.07 (m, 4H), 4.16 (m, 2H), 4.28 (m, 4H), 4.5 (m, 4H),4.7 (m, 2H), 6.04 (s, 2H), 6.26 (d, 2H), 6.5 (s, 2H), 7.1 (s, 2H), 7.2 (s, 2H), 7.9 (d, 1H), 8.1 (s, 2H); MS (FAB$^+$) mass calcd for $C_{174}H_{254}N_{34}O_{33}Co_2P_2S$ 3562, found 3561 $(M)^2$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) λ_{360} (ϵ = 38 700).

e-Acid Dimer (**31**): yield, 100 mg (50%); mp > 300 °C; ^{1}H NMR (MeOH-d_4) δ 0.43 (s, 6H), 1.17 (s, 8H), 1.3 (m, 20H), 1.35 (m, 22H), 1.43 (s, 10H), 1.53 (m, 8H), 1.7 (m, 6H), 1.87 (m, 6H), 2.0 (m, 4H), 2.13 (s, 10H), 2.25 (d, 10H), 2.52 (d, 16H), 3.16 (m, 6H), 3.29 (s, 10H), 3.37 (m, 6H), 3.6 (m, 4H), 3.73 (m, 2H), 3.9 (d, 2H), 4.07 (m, 4H), 4.16 (m, 2H), 4.26 (m, 4H), 4.5 (m, 4H),4.64 (m, 2H), 6.04 (s, 2H), 6.26 (d, 2H), 6.6 (s, 2H), 7.1 (s, 2H), 7.23 (s, 2H), 7.9 (d, 1H), 8.1 (s, 2H); MS (FAB$^+$) mass calcd for $C_{174}H_{254}N_{34}O_{33}Co_2P_2S$ 3562, found 3562 $(M)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (MeOH) λ_{360} (ϵ = 40 000).

Conjugation of Cyanocobalamin Monocarboxylic Acid–Trioxadiamine with Isophthaloyl Dichloride; Synthesis of 32. To a solution containing 300 mg (0.193 mmol) of **8** in 20 mL of DMF was added 30 μL of triethylamine. To that solution was added 19.5 mg (0.096 mmol) of isophthaloyl dichloride over a 10–15 min period. The reaction mixture was stirred at rt for 4–5 days, and 30 μL of triethylamine was added after each 24 h period. After evaporating to dryness, the solid was dissolved in 20 mL of a 1:1 methanol/H_2O mixture and was applied to a preparative reversed phase column (25 mm × 500 mm), which was eluted with the same solvent. The fractions containing the final product were evaporated to dryness.

b-Acid Dimer (**32**): yield, 100 mg (32%); mp 195–198 °C with decomposition; ^{1}H NMR (MeOH-d_4) δ 0.44 (s, 6H), 1.18 (s, 6H), 1.25 (d, 7H), 1.31 (t, 20H), 1.36 (s, 14H), 1.45 (s, 8H), 1.74 (m, 20H), 1.88 (d, 15H), 2.27 (s, 11H), 2.37 (m, 22H), 2.56 (d, 20H), 2.85 (s, 5H), 2.99 (s, 2H), 3.2 (m, 18H), 3.3 (m, 12H), 3.4 (m, 10H), 3.5 (s, 14H), 3.58 (s, 18H), 3.6 (s, 30H), 3.9 (d, 4H), 4.0 (d, 2H), 4.1 (d, 2H), 4.18 (d, 2H), 4.3 (m, 2H), 4.5 (m, 2H), 4.7 (m, 2H), 6.0 (s, 2H), 6.28 (s, 2H), 6.56 (s, 2H), 7.1 (s, 2H), 7.25 (s, 2H), 7.56 (m, 1H), 7.8 (d, 2H), 8.3 (s, 1H); MS (FAB$^+$) mass calcd for $C_{154}H_{220}N_{30}O_{36}Co_2P_2$ 3245, found 3245 $(M)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (H_2O) λ_{361} (ϵ = 33 900).

Conjugation of Cyanocobalamin Monocarboxylic Acid–Trioxadiamine with DiTFP Ester of Biotin–Aminocaproate Aminoisophthalate; Synthesis of 33 and 34. To a solution containing 300 mg (0.193 mmol) **8** or **10** in 15 mL of DMF was added 30 μL of triethylamine. To that solution was added 79 mg (97 mmol) of **20** over a 5–10 min period. The reaction mixture was stirred at rt for 3–4 days (HPLC monitored), adding 30 μL of triethylamine after each 24 h period. The reaction mixture was then evaporated to dryness. The solid residue was dissolved in 20 mL of a 1:1 methanol/H_2O mixture and applied to a preparative reversed phase column (25 mm × 500 mm), which was eluted with the same solvent. The fractions containing the product were evaporated to dryness.

b-Acid Dimer (**33**): yield, 160 mg (53%); mp 192–195 °C with decomposition; ^{1}H NMR (MeOH-d_4) δ 0.43 (s, 6H), 1.17 (s, 6H), 1.24 (d, 8H), 1.36 (d, 16H), 1.44 (s, 6H), 1.73 (m, 20H), 1.87 (m, 12H), 2.26 (s, 10H), 2.4 (m, 14H), 2.55 (m, 18H), 3.17–3.3 (m, 30H), 3.6 (m, 40H), 3.73 (m, 2H), 3.9 (m, 2H), 4.06 (m, 2H), 4.1 (m, 2H), 4.17 (d, 2H), 4.3 (m, 4H), 4.47 (m, 4H), 4.6 (m, 2H), 4.66 (m, 2H),6.03 (s, 2H), 6.27 (s, 2H), 6.55 (s, 2H), 7.12 (s, 2H), 7.25 (s, 2H), 7.9 (s, 1H), 8.16 (s, 2H); MS (FAB$^+$) mass calcd for $C_{170}H_{246}N_{34}O_{39}Co_2P_2S$ 3602, found 3602 $(M)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (H_2O) λ_{360} (ϵ = 35 300).

e-Acid Dimer (**34**): yield, 120 mg (40%); mp 235–239 °C with decomposition; ^{1}H NMR (MeOH-d_4) δ 0.43 (s, 6H), 1.17 (d, 6H), 1.24 (d, 8H), 1.36 (d, 16H), 1.43 (s, 6H), 1.73 (m, 20H), 1.87 (m, 12H), 2.26 (d, 10H), 2.35 (m, 14H), 2.55 (m, 18H), 3.17–3.3 (m, 30H), 3.6 (m, 40H), 3.73 (m, 2H), 3.88 (m, 2H), 4.07 (m, 2H), 4.1 (m, 2H), 4.18 (d, 2H), 4.3 (m, 4H), 4.47 (m, 4H), 4.57 (m, 2H), 4.65 (m, 2H),6.03 (s, 2H), 6.27 (s, 2H), 6.56 (s, 2H), 7.11 (s, 2H), 7.25 (s, 2H), 7.9 (s, 1H), 8.16 (s, 2H); MS (FAB$^+$) mass calcd for $C_{170}H_{246}N_{34}O_{39}Co_2P_2S$ 3602, found 3603 $(M + H)^+$; IR (KBr, cm^{-1}) 3400, 3200, 2950, 2060, 1660, 1570, 1490, 1060; UV (H_2O) λ_{360} (ϵ = 31 400).

Binding of Cobalamin Derivatives with rhTCII. Recombinant human transcobalamin II (rhTCII) was prepared as previously reported (*22*). Stock solutions of Cbl derivatives were prepared by dissolving 2 mg in 100–200 μL of DMSO, followed by dilution to 1.0 mL with H_2O. Quantification of the Cbl derivatives was accomplished by UV, based on the measured extinction coefficients at 360 nm (data provided with individual compounds). Thus, a 20 μL aliquot of the stock Cbl solution was diluted to 1 mL with H_2O, and the absorbance at 360 nm was measured. An aliquot of the original solution was diluted to obtain a 1 μM solution, which was further diluted to 0.1 μM for use in the assay.

rhTCII was partially purified on a cation exchange column as previously described (*23*). The rhTCII was diluted in PBS containing 0.025% HSA to bind approximately 10 pmol of CN-Cbl (**1**)/mL. Aliquots of 100 μL of the rhTCII solution were added to tubes containing 0.01 pmol of [^{57}Co]-**1** and 0.1–30 pmol of unmodified **1** or Cbl derivatives **2**–**18**. The solution volume in each tube was adjusted to 1.0 mL with PBS, and the samples

Table 1. Binding of Cbl Dimers with rhTCII Relative to CN-Cbl, 1

cobalamin (compound no.)	50% binding inhibition for [^{57}Co]CN-Cbl[a]	% relative rhTCII binding
1	1	100
21	111	0.9
22	284	0.35
23	5	20
24	52	1.9
25	6	16
26	1.8	56
30	26	3.8
31	4.5	22
32	23	4.3
33	23	4.3
34	1.8	56

[a] The binding inhibition quantity is that amount (pmol) of Cbl derivative required to inhibit by 50% the binding of 0.01 pmol of [^{57}Co]CN-Cbl and was obtained by dividing 100 by the 50% binding inhibition quantity.

were incubated at rt for 1 h. The protein-bound [^{57}Co]-**1** was then separated from free [^{57}Co]-**1** by adsorption to hemoglobin-coated charcoal (*24*). The amount of radioactivity in each fraction was determined in a gamma counter. The decrease in binding of [^{57}Co]-**1** in the presence of various amounts of a Cbl derivative was calculated and graphed (Figure 2). The quantities of each derivative required to inhibit the binding of [^{57}Co]CN-Cbl to rhTCII by 50% was determined, and the apparent affinities of the compounds relative to CN-Cbl were calculated (Table 1).

RESULTS AND DISCUSSION

There are only a few reported examples of the preparation of Cbl dimers. In one example, it is proposed that Co–S–S–Co Cbl dimers are prepared from reaction with hydrosulfide ion (*25*). More recently, cobalt oligomethylene bridged dimers [Co–$(CH_2)_n$–Co, where $n = 4–6$] have been prepared as potential "latent alkanediyl diradicals" (*26*). Since our ultimate goal is to obtain Cbl derivatives that could be used as therapeutic pharmaceuticals, the light sensitivity and instability of previously described cobalt-linked dimers made them unsuitable. Therefore, we directed our efforts to the synthesis of novel new Cbl dimers that are designed to be stable to degradation. The synthetic approach chosen for preparation of stable Cbl dimers was to employ a di- or trifunctional cross-linking reagent that could couple two Cbl corrin ring carboxylates through linking moieties, as depicted in Figure 1. While bioactive Cbl dimers may be obtained with a difunctional cross-linking reagent, the desire to incorporate other functionalities into the dimers led us to consider a trifunctional reagent. This cross-linking arrangement allows for attachment of other chemical moieties with specific biological properties without having to make a second attachment on the Cbl moiety. Since we were interested in the possibility of binding two TCII molecules to the same Cbl dimer, the distance between the two Cbl moieties was considered to be critical for formation of such a complex. The linker molecules employed have 14–15 atoms in the chain, and linking of two of these with the isophthalic acid cross-linking moiety provides a 41–42 Å (fully extended) distance between the Cbls. This distance was thought to be adequate to bind two TCII molecules.

Synthesis of Cobalamin Dimers and Precursor Molecules. Our approach to preparing the Cbl dimers was to synthesize the diamino-linker adduct of Cbl carboxylates first and then couple the two adducts with the cross-linking reagent. This approach was chosen because the Cbl diamino-linker adducts had been previously prepared for related studies (*12*, *13*). The initial dimeric Cbls synthesized (Chart 2) incorporated diaminododecane adducts **5**–**7** (Chart 1) with isophthalate cross-linking moieties. The alkylamino derivatives **5**, **6**, and **7** were prepared by conjugation of the Cbl-carboxylates **2**, **3**, or **4** with diaminododecane using the water soluble carbodiimide, EDC,[3] in DMF. The carboxylates were prepared from mild acid hydrolysis of CN-Cbl, **1**, followed by careful separation of the isomers on a preparative LC system employing an aminopropyl-silica column (*12*). Synthesis of the isomeric Cbl dimers **21**–**23** was accomplished by reacting one of the diaminododecane adducts, **5**, **6**, or **7**, with isophthaloyl dichloride. All three isomeric derivatives (e.g. *b*, *d*, and *e*) were prepared to compare the TCII binding of dimeric molecules with their monomeric counterparts.

Radioiodination of Cbl dimers was initiated to evaluate the biological properties of these compounds. We have previously shown that, while direct radioiodination of Cbl does not yield the desired product, radioiodination of Cbls could be accomplished by conjugation with arylstannane moieties (*13*). In general, incorporation of high specific activity radioiodine into a benzoyl moiety is readily achieved from the corresponding trialkylstannyl benzoate intermediates (*18*). One approach to obtaining radioiodinated dimers was to prepare them from stannylbenzoyl adducts of aminoisophthalate cross-linked Cbls. Thus, *p*-(tri-*n*-butylstannyl)benzoyl-containing isophthaloyl dimers **27**–**29** were prepared to be used for incorporation of radioiodine into the dimers, and *p*-iodobenzoyl-containing isophthaloyl dimers **24**–**26** were prepared as HPLC standards for radioiodinated Cbl derivatives.

Prior to the synthesis of the Cbl dimers **24**–**29**, the requisite activated isophthaloyl derivatives had to be prepared. The synthesis of ditetrafluorophenyl (TFP) esters of the iodobenzoyl and stannylbenzoyl adducts of 5-aminoisophthalic acid, **13** and **14**, is shown in Scheme 1. Conjugation of *p*-iodobenzoyl chloride with 5-aminoisophthalic acid gave a nearly quantitative yield of the crude adduct, **12**. Preparation of the di-TFP ester **13** was accomplished in 43% yield by reacting **12** with DCC and TFP-OH in ethyl acetate. Conversion of the aryl iodide, **13**, into the aryl stannane, **14**, was accomplished in 62% yield using bis(tributyltin) and palladium catalyst.

Biotinylated Cbl dimers were of interest as reagents for analytical studies. Therefore, Cbl dimers **30** and **31** were prepared. Rather than preparing all three isomeric biotinylated derivatives, only the Cbls containing *b*- and *e*-propionic acid side chains (**2** and **4**) were employed for cross-linking reactions. Indeed, we were primarily interested in the *e*-isomer of the biotinylated dimer as it was expected to have the highest binding with TCII, but since the Cbl *e*-carboxylate was available in small quantities, initial development of cross-linking reaction conditions was conducted with the more abundant *b*-carboxylate, **2**. Prior to cross-linking the aminoalkyl-Cbls **5** or **7**, a carboxylate activated–biotinylated aminoisophthalic acid derivative, **20**, had to be prepared. Although *N*-hydroxysuccinimido ester of biotin–aminocaproate was commercially available,[4] the high cost of this reagent led us to synthesize the corresponding TFP ester, **18** (Scheme 2). Reaction of **18** with 5-aminoisophthalic acid provided

[3] EDC is also referred to as EDAC (Sigma Chemical Co., St. Louis, MO); 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide.

[4] For example: sulfosuccinimido-6-(biotinamido)hexanoate is available from Pierce (Rockford, IL) as NHS-LC-Biotin, and biotinamidocaproate *N*-hydroxysuccinimide ester is available from Sigma Chemical Co. (St. Louis, MO).

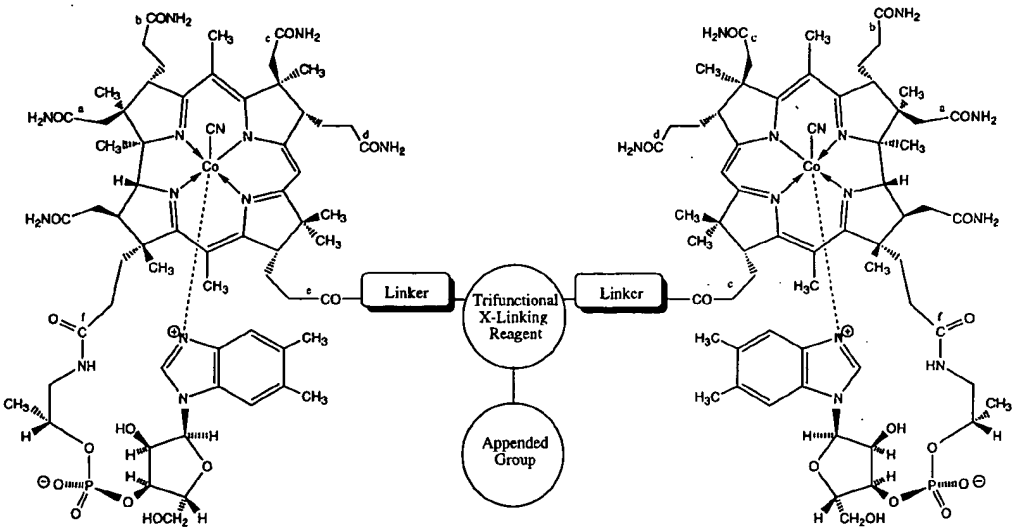
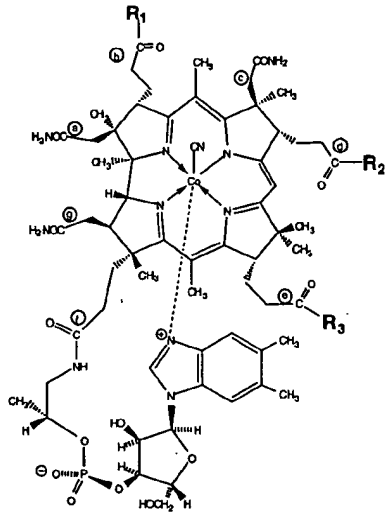


Figure 1. General schematic representation of CN-Cbl dimers. The dimers contain two Cbl moieties, two linking moieties, a cross-linking moiety, and, where desired, an appended group. This example depicts corrin ring *e*-propionamide attachment.

Chart 1. Structures of Cyanocobalamin Diamino Spacer Adducts

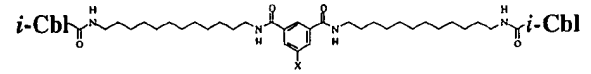


1 $R_1 = NH_2$; $R_2 = NH_2$; $R_3 = NH_2$
2 $R_1 = OH$; $R_2 = NH_2$; $R_3 = NH_2$
3 $R_1 = NH_2$; $R_2 = OH$; $R_3 = NH_2$
4 $R_1 = NH_2$; $R_2 = NH_2$; $R_3 = OH$
5 $R_1 = HN(CH_2)_{12}NH_2$; $R_2 = NH_2$; $R_3 = NH_2$
6 $R_1 = NH_2$; $R_2 = HN(CH_2)_{12}NH_2$; $R_3 = NH_2$
7 $R_1 = NH_2$; $R_2 = NH_2$; $R_3 = HN(CH_2)_{12}NH_2$
8 $R_1 = HN(CH_2)_3(OCH_2CH_2)_2O(CH_2)_3NH_2$; $R_2 = NH_2$; $R_3 = NH_2$
9 $R_1 = NH_2$; $R_2 = HN(CH_2)_3(OCH_2CH_2)_2O(CH_2)_3NH_2$; $R_3 = NH_2$
10 $R_1 = NH_2$; $R_2 = NH_2$; $R_3 = HN(CH_2)_3(OCH_2CH_2)_2O(CH_2)_3NH_2$

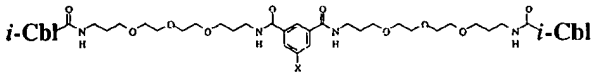
19 in 65% yield, and further conversion to **20** using the transesterification reagent tetrafluorophenyl trifluoroacetate (TFP-OCOCF$_3$) (*21*) was accomplished in 86% yield.

It became apparent after the synthesis of Cbl dimers **21**–**31** that they had limited water solubility. Fortunately, very low concentrations of Cbls are required to obtain biological data with these compounds, permitting *in vitro* evaluations to be conducted. However, the increased lipophilicity of the compounds was considered to be a potential problem with nonspecific binding and could ultimately make formulation of a pharmaceutical more difficult. Therefore, a method of water solubilization for the dimers was sought. The favored approach to solving this problem was to incorporate a more water soluble linking moiety. Thus, commercially available 4,7,10-trioxa-1,13-tridecanediamine was conjugated with two isomeric Cbl carboxylates, **2** and **4**, to yield Cbl adducts **8** and **10**. This linking arm is nearly the same length as the diaminododedane linker, resulting in distances between Cbl carboxylates of 41.6 Å for **23** (and related compounds) and 42.6 Å for **32** (and related compounds). The reason for preparing only the *b*- and *e*-isomers was based on availability of carboxylates as described previously. Conjugation of **8** with isophthaloyl dichloride provided Cbl dimer, **32**. Importantly, that compound was more soluble in water than its diaminododecane counterpart. Due to the anticipated increased water solubility of the simple isophthalate cross-linked molecules, the biotin dimers **33** and **34** were synthesized. The biotinylated Cbl dimers **33** and **34** were synthesized in 40–53% yield from the reaction of **8** or **10** with the

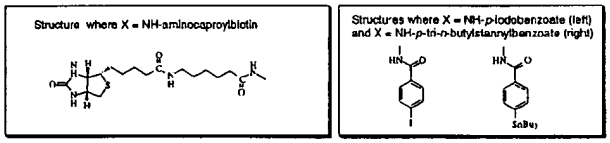
Chart 2. Cyanocobalamin Dimers Synthesized[a]



21 *i* = *b*-; X = H
22 *i* = *d*-; X = H
23 *i* = *e*-; X = H
24 *i* = *b*-; X = NH-*p*-iodobenzoate
25 *i* = *d*-; X = NH-*p*-iodobenzoate
26 *i* = *e*-; X = NH-*p*-iodobenzoate
27 *i* = *b*-; X = NH-*p*-tri-*n*-butylstannylbenzoate
28 *i* = *d*-; X = NH-*p*-tri-*n*-butylstannylbenzoate
29 *i* = *e*-; X = NH-*p*-tri-*n*-butylstannylbenzoate
30 *i* = *b*-; X = NH-aminocaproylbiotin
31 *i* = *e*-; X = NH-aminocaproylbiotin

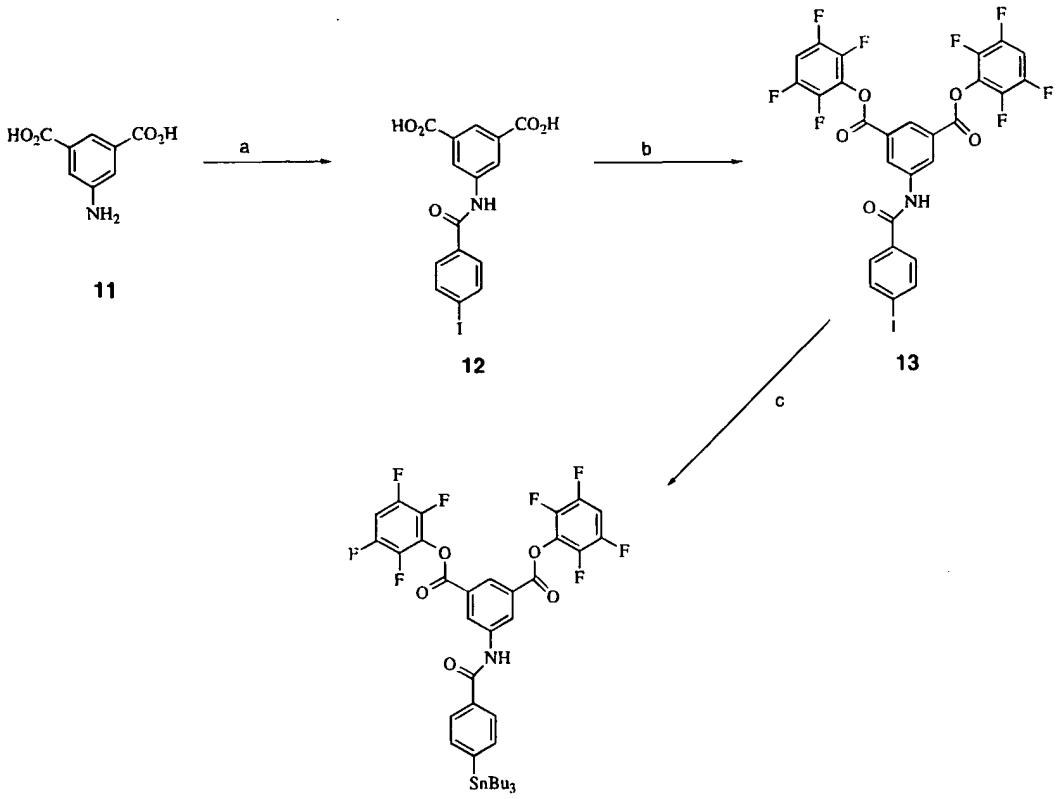


32 *i* = *b*-; X = H
33 *i* = *b*-; X = NH-aminocaproylbiotin
34 *i* = *e*-; X = NH-aminocaproylbiotin



[a] *i*-Cbl are isomers of Cbl carboxylates **2** (*b*-), **3** (*d*-), or **4** (*e*-).

Scheme 1. Synthesis of Isophthalate DiTFP Ester Containing Iodobenzoate or Stannylbenzoate



[a] 1 N NaOH/iodobenzoyl chloride/0 °C. [b] TFP-OH/DCC/3 days. [c] Bu_3Sn_2/$(Ph_3P)_4Pd(O)$/toluene/80 °C.

aminoisophthalate di-TFP ester **20**. Again, the resulting Cbl dimers were found to be more soluble in aqueous medium than their diaminododecane counterparts, **30** and **31**.

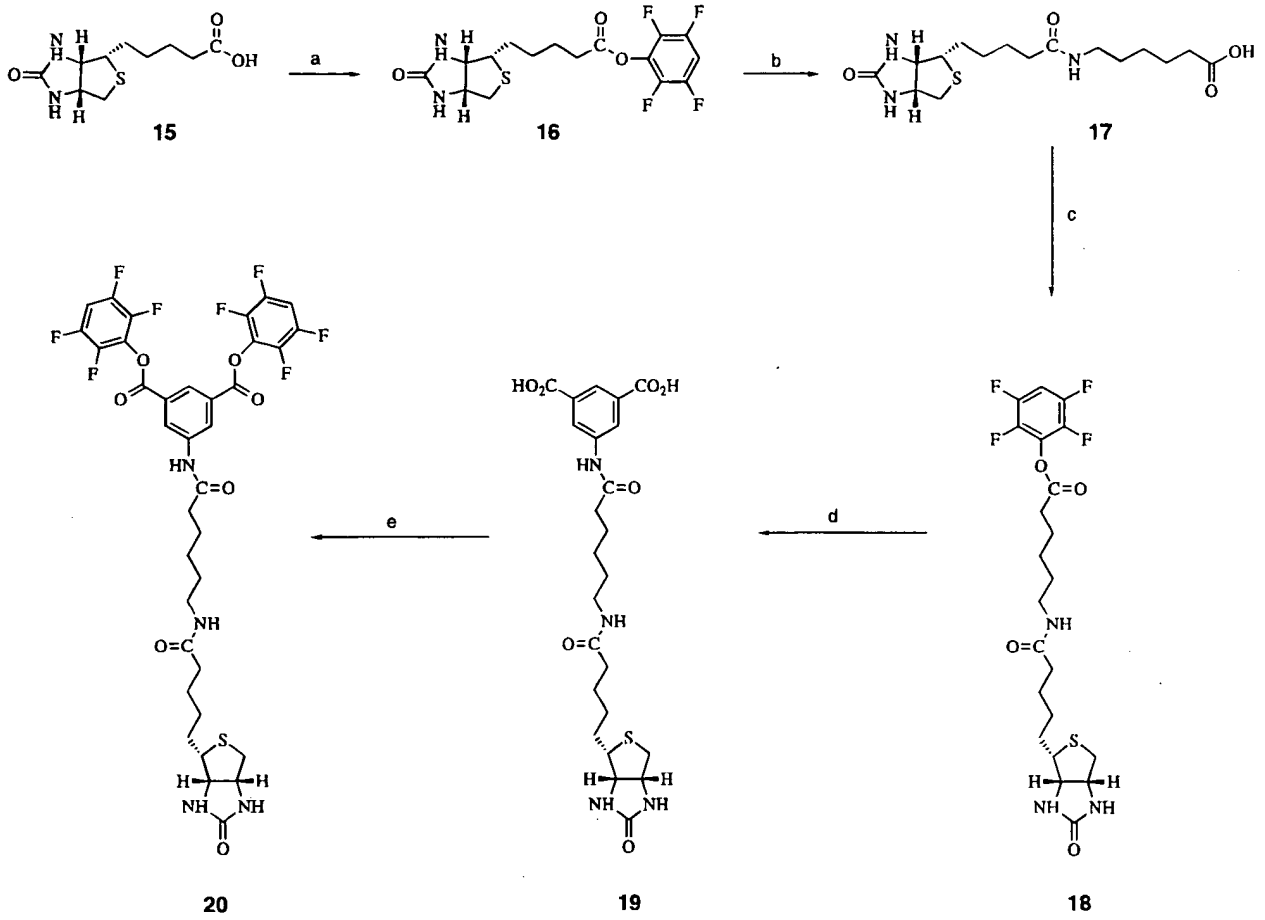
Iodination Reactions. Iodination and radioiodination of Cbl derivatives containing arylstannanes must be conducted under (near) neutral conditions or a side reaction for the formation of *c*-lactone can predominate (*15*). Attempts at iodination of HPLC purified stannyl derivatives **27**–**29** in MeOH using NaI and *N*-chlorosuccinimide (NCS)[5] over a 30 min period resulted in no change in the starting material. Attempts at iodination using NaI/NCS in 5% HOAc/MeOH also did not alter the starting material. Because there was a concern that the iodide ion was somehow being inactivated toward oxidation with NCS (e.g. being bonded to the Co in the corrin ring), ICl was investigated as the iodination reagent. Attempts to iodinate **29** with ICl in MeOH over a 30 min period also failed to change the starting material; however, reaction of ICl in 5% HOAc/MeOH resulted in conversion to another compound. Unfortunately, the new compound was not the desired iodinated product as its HPLC retention time was not the same as that of **26**. Although the data obtained were insufficient to determine the nature of the new compound, it seems likely that it is the *c*-lactone derivative of **26**. An evaluation of the radioiodination of **29** also did not provide the desired compound.

The lack of conversion of the arylstannanes (**27**–**29**) to aryliodides (**24**–**26**) was surprising. Under the reaction conditions employed, no previous iodinations of arylstannyl-derivatized compounds have failed to affect rapid (generally <1 min) and nearly quantitative substitution of the stannyl group. This failure to react with electrophilic iodine could be due to unique nature of the Cbls synthesized. The mass spectral data and chemical shifts of butyl and aryl protons in the NMR spectra indicated that the arylstannane is present in the dimer. However, the HPLC retention time of the arylstannane derivatives **27**–**29** was not as expected. Generally on reversed phase HPLC, aryltri-*n*-butylstannyl derivatives are retained much longer than the corresponding aryliodide derivatives. This was not the case with compounds **27**–**29**, as they had shorter retention times (8.6–9.0 min) than the corresponding aryliodides **24**–**26** (14.1–14.2 min). Since the spectral data suggest that the compounds are as depicted in Table 2, it is very difficult to understand how this increased polarity is obtained in the arylstannyl Cbl dimers. We can only hypothesize that the lipophilic linker arms and the lipophilic arylstannane are somehow compressed between the more hydrophilic Cbl moieties, perhaps causing a shift in the benzimidazole rings or other portion of the molecule, making it more hydrophilic. It is anticipated that we will be able to obtain more information on the structures through a crystal structure determination of the aryliodo derivative **26**. An attempt to obtain the crystal structure of **26** is underway.

Competitive Binding with TCII. Cbl dimers were evaluated for their binding to rhTCII in a competitive assay that measures the binding relative to CN-Cbl (*12*). The assay employed partially purified rhTCII as the binding protein, and high specific activity (e.g. 200 μCi/

[5] These reagents were used because the conditions mimic the radioiodination reaction conditions where NCS oxidizes Na[*I]I to an electrophilic species *in situ*.

Scheme 2. Synthesis of Isophthalate DiTFP Ester Containing Aminocaproylbiotin Substituent



[a] DCC/DMF/TFP/0 °C. [b] Aminocaproic acid/Et_3N. [c] DMSO/Et_3N/TFP-OTFA. [d] DMF/Et_3N/5-aminoisophthalic acid/8 days. [e] DMF/Et_3N/TFP-OTFA.

μg) [^{57}Co]CN-Cbl as the tracer. The assay measures the decrease in the binding of [^{57}Co]CN-Cbl in the presence of increasing concentrations of a Cbl dimer. The competitive binding curves for Cbl dimers are shown in Figure 2. The binding curves for the four isophthalate cross-linked dimers, **21**–**23** and **32**, relative to CN-Cbl, **1**, are shown in Figure 2A. The data show that the *e*-isomer, **23**, binds more avidly than the *b*-isomer, **21**, or the *d*-isomer, **22**. It is interesting to note that the compounds of **21**–**23** appear to bind less avidly than the corresponding monomeric Cbls **5**–**7**, previously tested (*12*). The differences observed could be due to the decreased water solubility of the dimers compared to the monomers. Indeed, water solubility appears to affect Cbl binding as the more water soluble trioxadiamine-linked Cbl-*b*-dimer, **32**, bound more avidly than the diaminododecane Cbl-*b*-dimer, **21**.

Evaluation of iodobenzoyl-containing Cbl dimers, **24**–**26**, provided similar results to those observed for the other diaminododecane-containing dimers **21**–**23** (Figure 2B). Again, only the *e*-isomer, **26**, bound competitively with [^{57}Co]-**1**, and its binding appeared to be reduced from that of the monomeric counterpart **7**. Binding assays were not conducted on the stannylbenzoate-containing dimers **27**–**29** as these compounds were only to be used as intermediates to prepare (radio)iodinated derivatives **24**–**26**.

The results obtained for the biotinylated derivatives **30**, **31**, **33**, and **34** are shown in Figure 2C. Binding of the water-solubilized *b*- and *e*-isomers, **33** and **34**, to rhTCII is similar to the isomeric monomers **5** and **7**. The *e*-isomer, **31**, which contains the less water soluble diaminododecane linking moiety, does not bind as competitively as the corresponding more water soluble *e*-isomer, **34**. Again, this difference may be due to the lower water solubility of **31**. Relative binding data for the Cbl dimers are provided in Table 1. From the data, the decrease in binding (from 100%) and the effect of various modifications in the Cbl dimers on binding can be readily assessed. It is interesting to note that substitution on the isophthalate moiety appears to have little effect on TCII binding, whereas there is a significant improvement in binding with the addition of the trioxadiamine linker.

One of the questions we attempted to address in this investigation was whether a Cbl dimer could bind two TCII protein molecules. On the basis of the data obtained from the competitive binding assay, it appears that under the assay conditions (e.g. excess Cbl), only one Cbl moiety in the dimers bound with rhTCII. However, unless large quantities (e.g. micrograms–milligrams) of a dimer were injected in patients, the amount of TCII should be in excess even when considering endogenous Cbls (*8*, *29*). The results obtained in the competitive binding assay are not surprising as it seems likely that binding of a Cbl dimer with the one TCII molecule could occur at a rate similar to a monomer but that the second TCII molecule would have to combine with a Cbl that was highly sterically hindered from the side that had bound TCII. This would suggest that binding with a second TCII

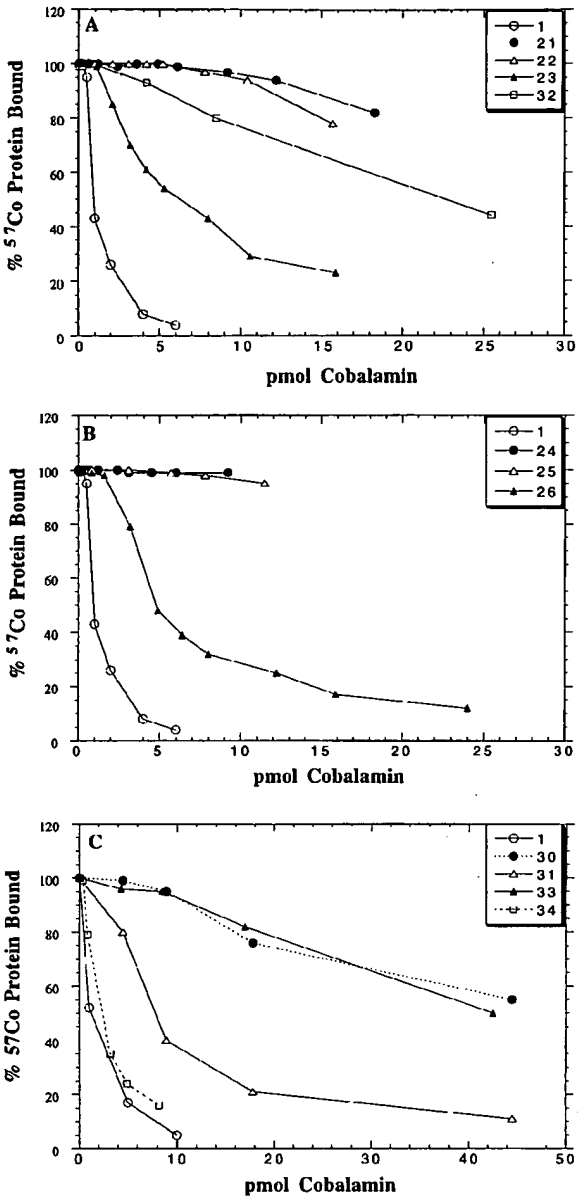


Figure 2. Competitive binding curves for [^{57}Co]CN-Cbl, **1**, and Cbl dimers **21**–**26** and **30**–**34** with rhTCII. To obtain the data, a quantity of rhTCII that would bind approximately **1** pmol of CN-Cbl was mixed with 0.01 pmol of [^{57}Co]-**1** and 0.01–30 pmol of unmodified **1** or Cbl dimer. The mixture was diluted with PBS/ HSA and incubated at rt for 1 h. Free Cbl was adsorbed onto hemoglobin-coated charcoal, and the protein-bound radioactivity in the supernatant fraction was measured. The amount of protein-bound ^{57}Co versus the amount of Cbl derivative present is plotted in panels A–C. Panel A (top) compares the competitive binding of the isophthalate cross-linked Cbl dimers that employed diaminododecane linker (**21**, **22**, **23**) and trioxadiamine linker (**32**) with CN-Cbl (**1**). Panel B (middle) compares the competitive binding of *p*-iodobenzoyl-5-aminoisophthalate cross-linked Cbl dimers (**24**, **25**, **26**) with **1**. Panel C (bottom) compares the competitive binding of biotin containing Cbl dimers that employed the diaminododecane linker (**30**, **31**) and the trioxadiamine linker (**33**, **34**) with **1**.

molecule might occur at a much slower rate. Determination of whether the dimers described herein bind with two rhTCII molecules when the TCII is in excess has proven to be difficult with *partially purified* rhTCII. Thus, future studies will be directed at obtaining radioiodinated Cbl dimers to improve sensitivity of detecting Cbl/TCII complexes by electrophoresis and size exclusion HPLC.

SUMMARY

Fourteen isophthalate cross-linked Cbl dimers have been synthesized. To our knowledge, the reported compounds represent the first preparation of Cbl dimers that are coupled at a position other than at the Co atom. The Cbl dimers were prepared by cross-linking with carboxylate-activated derivatives of isophthalic acid or derivatized aminoisophthalic acid. To provide a distance between the Cbl moieties such that two TCII proteins might bind a Cbl dimer, linking molecules were incorporated between the Cbl moieties and the isophthalic acid moieties. The linker first evaluated, 1,12-diaminododecane, was found to decrease the water solubility of the Cbls considerably; therefore, a more water soluble linker, 4,7,-10-trioxa-1,13-tridecanediamine, was also evaluated. The trioxatridecanediamine improved the water solubility significantly. In the investigation, Cbl dimers were evaluated for their binding with rhTCII. Analysis of the binding data shows that most of the Cbl dimers bound less avidly to rhTCII than CN-Cbl. As expected, the Cbl *e*-carboxylate derived dimers bound most avidly of the three isomeric carboxylates studied. The more water soluble dimers bound rhTCII more avidly than their aliphatic counterparts, resulting in binding that was nearly equivalent to that of CN-Cbl for the *e*-carboxylate isomer.

In this initial investigation, the Cbl dimers synthesized have provided basic information about their chemistry and binding to TCII. Studies are planned to prepare additional water soluble Cbl dimers and to obtain radioiodinated Cbl dimers. Once a radioiodinated Cbl dimer is obtained, the question of whether it can bind two TCII molecules will be assessed. It is anticipated that more highly derivatized Cbl dimers will be needed to be effective in Cbl/TCII cell surface receptor depletion, but all Cbl derivatives synthesized will be evaluated for potency as antiproliferative agents in *in vitro* assays. The data obtained from *in vitro* analyses will be reported elsewhere.

ACKNOWLEDGMENT

We thank Dr. Ross Lawrence (Medicinal Chemistry Department, University of Washington) for his efforts in obtaining mass spectral data on the Cbl derivatives. We are grateful for the financial support provided by Receptagen Corp. to conduct these studies.

Supporting Information Available: HPLC chromatograms and ^{1}H NMR spectra of trioxadiamine Cbl derivatives **8**–**10**, Cbl dimers **21**–**34**, iodobenzoyl and stannylbenzoyl aminoisophthalates **13** and **14**, and biotinylated derivatives **18**, **19**, and **20** (39 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

(1) Banerjee, R. V., and Matthews, R. G. (1990) Cobalamin-Dependent Methionine Synthase. *FASEB J. 4*, 1450–1459.
(2) Weissbach, H., and Taylor R. T. (1968) Metabolic Role of Vitamin B_{12}. *Vitamins and Hormones* (R. S. Harris, I. G. Wool, and J. A. Loraine, Eds.) Vol. 26, pp 395–412, Academic Press, New York.
(3) Myasishcheva, N. V. (1990) Cobalamin metabolism in acute lymphoblastic leukaemia in children. *Biomedicine and Physiology of Vitamin B_{12}* (J. C. Linnell and H. R. Bhatt, Eds.) pp 193–198, Children's Medical Charity, London.
(4) Faludy, J. E., and Linnell, J. C. (1990) Cobalamin metabolism in human leukæmia cell lines: a pilot study. *Biomedicine*

and Physiology of Vitamin B_{12} (J. C. Linnell and H. R. Bhatt, Eds.) pp 199–205, Children's Medical Charity, London.
(5) Lassen, H. C. A., and Kristensen, H. S. (1959) Remission in chronic myeloid leukaemia following prolonged nitrous oxide inhalation. *Danish Med. Bull. 6*, 252–254.
(6) Eastwood, D. W., Green, C. D., Lambdin, M. A., and Gardner, R. (1963) Effect of nitrous oxide on the white-cell count in leukemia. *New Engl. J. Med. 268*, 297–299.
(7) Hall, A. C. (1990) The role of Transcobalamin II in the cellular uptake of cobalamin. *Biomedicine and Physiology of Vitamin B_{12}* (J. C. Linnell and H. R. Bhatt, Eds.) pp 239–253, Children's Medical Charity, London.
(8) Seetharam, B. (1994) Gastrointestinal Absorption and Transport of Cobalamin (Vitamin B_{12}). *Physiology of the Gastrointestinal Tract* (L. R. Johnson, Ed.) pp 1997–2026. Raven Press, New York.
(9) Quadros, E. V., Sai, P., and Rothenberg, S. P. (1994) Characterization of the Human Placental Membrane Receptor for Transcobalamin II–Cobalamin. *Arch. Biochem. Biophys. 308*, 192–199.
(10) Jacobsen, D. W., Amagasaki, T., and Green, R. (1990) Synthesis and recycling of the Transcobalamin II receptor. *Biomedicine and Physiology of Vitamin B_{12}* (J. C. Linnell and H. R. Bhatt, Eds.) pp 293–306, Children's Medical Charity, London.
(11) De Duve, C., de Barsy, T., Poole, B., Trouet, A., Tulkens, P., and Van Hoof, F. (1974) Lysosomotropic Agents. *Biochem. Biopharmacol. 23*, 2495–2531.
(12) Pathare, P. M., Wilbur, D. S., Heusser, S., Quadros, E. V., McLoughlin, P., and Morgan, A. C. (1996) Synthesis of Cobalamin-Biotin Conjugates Which Vary in Position of Coupling. An Evaluation of the Location of Conjugated Groups on Transcobalamin II Binding. *Bioconjugate Chem. 7*, 217–232.
(13) Wilbur, D. S., Hamlin, D. K., Pathare, P. M., Heusser, S., Vessella, R. L., Buhler, K. R., Stray, J. E., Daniel, J., Quadros, E. V., McLoughlin, P., and Morgan, A. C. (1995) Synthesis and *nca*-Radioiodination of Arylstannylcobalamin Conjugates. Evaluation of Aryliodocobalamin Conjugate Binding to Transcobalamin II and Biodistribution in Mice. *Bioconjugate Chem. 7*, 461–474.
(14) Bonnett, R. (1982) Reactions of the Corrin Macrocycle. *B_{12}: Vol. 1—Chemistry* (D. Dolphin, Ed.) pp 201–243, Wiley, New York.
(15) Morley, C. G. D., Blakley, R. L., and Hogenkamp, H. P. C. (1968) Analogs of Deoxyadenosylcobalamin with Alterations in a Side Chain of the Corrin Ring. *Biochemistry 7*, 1231–1239.
(16) Kenley, J. S., Leighton, M., and Bradbeer, C. (1978) Transport of Vitamin B_{12} in *Escherichia coli*. Corrinoid Specificity of the Outer Membrane Receptor. *J. Biol. Chem. 253*, 1341–1346.
(17) Toraya, T., Krodel, E., Mildvan, A. S., and Abeles, R. H. (1979) Role of Peripheral Side Chains of Vitamin B_{12} Coenzymes in the Reaction Catalyzed by Dioldehydrase. *Biochemistry 18*, 417–426.
(18) Wilbur, D. S. (1992) Radiohalogenation of Proteins: An Overview of Radionuclides, Labeling Methods, and Reagents for Conjugate Labeling. *Bioconjugate Chem. 3*, 433–470.
(19) Kabalka, G. W., and Varma, R. S. (1989) The Synthesis of Radiolabeled Compounds Via Organometallic Intermediates. *Tetrahedron 45*, 6601–6621.
(20) Wilbur, D. S., Hamlin, D. K., Vessella, R. L., Stray, J. E., Buhler, K. R., Stayton, P. S., Klumb, L. H., Pathare, P. M., and Weerawarna, S. A. (1996) Antibody Fragments in Tumor Pretargeting. Evaluation of Biotinylated Fab' Co-Localization with Recombinant Streptavidin and Avidin. *Bioconjugate Chem. 7*, 689–702.
(21) Gamper, H. B., Reed, M. W., Cox, T., Virosco, J. S., Adams, A. D., Gall, A. A., Scholler, J. K., and Meyer, R. B. (1993) Facile preparation of nuclease resistant 3' modified oligodeoxynucleotides. *Nucleic Acids Res. 21*, 145–150.
(22) Quadros, E. V., Sai, P., and Rothenberg, S. P. (1993) Functional human transcobalamin II isoproteins are secreted by insect cells using the Baculovirus expression system. *Blood 81*, 1239–1245.
(23) Quadros, E. V., Rothenberg, S. P., Pan, Y. Ch. E., and Stein, S. (1986) Purification and molecular characterization of human transcobalamin II. *J. Biol. Chem. 261*, 15455–15460.
(24) Lau, K. S., Gottlieb, C., Wasserman, L. R., and Herbert, V. (1965) Measurement of serum vitamin B_{12} level using radioisotope dilution and coated charcoal. *Blood 26*, 202–214.
(25) Toohey, J. I. (1993) Hydrosulfide Derivatives of Cobalamins. *J. Inorg. Biochem. 49*, 189–199.
(26) Krautler, B., Derer, T., Liu, T., Muhlecker, W., Puchberger, M., Gruber, K., and Kratky, C. (1995) Oligomethylene-Bridged Vitamin B_{12} Dimers. *Angew. Chem., Int. Ed. Engl. 34*, 84–86.
(27) Linnell, J. C. (1975) The fate of Cobalamins *in vivo*. In *Cobalamin. Biochemistry and Pathophysiology* (B. M. Babior, Ed.) Chapter 6, pp 287–333, Wiley, New York.

BC970003+

INTER LIBRARY LOAN REQUEST FORM

QS583-A37

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

8901601 BIOSIS NO.: 199396053102
Effects of vitamin B-12 analogues with alternations in the side chains of the corrin ring on urinary methylmalonate excretion in vitamin B-12-deficient rats.
AUTHOR: Saido Hisako; Watanabe Fumio; Tamura Yoshiyuki; Manai Yasuo; Nakano Yoshihisa(a)
AUTHOR ADDRESS: (a)Dep. Agric. Chem., Univ. Osaka Prefecture, Sakai, Osaka 593**Japan
JOURNAL: Bioscience Biotechnology and Biochemistry 57 (4):p607-610 1993
ISSN: 0916-8451
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: To study how much the side chains of the corrin ring of vitamin B-12 are involved in the physiological roles of the vitamin, five vitamin B-12 analogues (cyanocobalamin-b-monocarboxylate, cyanocobalamin-d-monocarboxylate, cyanocobalamin-e-monocarboxylate, cyano-13-epicobalamin, and cyanocobalamin(c-lactam)) with alternations in the side chains were synthesized chemically and then administered orally and intravenously to vitamin B-12-deficient rats. Male rats fed a vitamin B-12-deficient diet for 11 wk developed a severe vitamin B-12 deficiency with a high urinary methylmalonate excretion (223.8 +- 136.2 mu-mol/d) and -97% (1.2 +- 0.7 ng/g tissue) lower hepatic vitamin B-12 content. Oral and intravenous administration of cyanocobalamin-b-,-d-, and -e-monocarboxylates and cyano-13-epicobalamin could not improve the severe vitamin B-12-deficient status of the rats, indicating that the b-, d-, and e-propionamide side chains of the corrin ring of vitamin B-12 are important in the absorption, transport, and function of the vitamin in rats. Urinary methylmalonate excretion of the rats that were intravenously administered cyanocobalamin(clactam) increased twice as much as those of the other analogue-supplemented rats, suggesting that cyanocobalamin(c-lactam) act as a powerful **Cbl-antagonist**. The results also indicate that mammalian cells do not contain a system for synthesizing complete vitamin B-12 from these analogues.

Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

Biosci. Biotech. Biochem., 57 (4), 607–610, 1993

Effects of Vitamin B_{12} Analogues with Alternations in the Side Chains of the Corrin Ring on Urinary Methylmalonate Excretion in Vitamin B_{12}-Deficient Rats

Hisako SAIDO, Fumio WATANABE,* Yoshiyuki TAMURA,* Yasuo MANAI, and Yoshihisa NAKANO†

Department of Agricultural Chemistry, University of Osaka Prefecture, Sakai, Osaka 593, Japan
** Laboratory of Nutrition and Food Science, Hagoromo-gakuen College, 1–89–1 Hamadera-minamimachi, Sakai, Osaka 592, Japan*
Received October 2, 1992

To study how much the side chains of the corrin ring of vitamin B_{12} are involved in the physiological roles of the vitamin, five vitamin B_{12} analogues (cyanocobalamin-*b*-monocarboxylate, cyanocobalamin-*d*-monocarboxylate, cyanocobalamin-*e*-monocarboxylate, cyano-13-epicobalamin, and cyanocobalamin(*c*-lactam)) with alternations in the side chains were synthesized chemically and then administered orally and intravenously to vitamin B_{12}-deficient rats. Male rats fed a vitamin B_{12}-deficient diet for 11 wk developed a severe vitamin B_{12} deficiency with a high urinary methylmalonate excretion (223.8 ± 136.2 μmol/d) and ~97% (1.2 ± 0.7 ng/g tissue) lower hepatic vitamin B_{12} content. Oral and intravenous administration of cyanocobalamin-*b*-,-*d*-, and -*e*-monocarboxylates and cyano-13-epicobalamin could not improve the severe vitamin B_{12}-deficient status of the rats, indicating that the *b*-, *d*-, and *e*-propionamide side chains of the corrin ring of vitamin B_{12} are important in the absorption, transport, and function of the vitamin in rats. Urinary methylmalonate excretion of the rats that were intravenously administered cyanocobalamin(*c*-lactam) increased twice as much as those of the other analogue-supplemented rats, suggesting that cyanocobalamin(*c*-lactam) act as a powerful Cbl-antagonist. The results also indicate that mammalian cells do not contain a system for synthesizing complete vitamin B_{12} from these analogues.

The relationship between the structure of vitamin B_{12} (Cbl) and its functions has been studied extensively in some Cbl-dependent enzymes and -binding proteins.[1–3] The side chains of the corrin ring of Cbl have been reported to be essential for molecular recognition in the binding of Cbl to Cbl-dependent enzymes and Cbl-binding proteins.[2–4] Mammalian livers contain MeCbl-dependent methionine synthase (EC 2.1.1.13) involved in methionine biosynthesis and AdoCbl-dependent methylmalonyl-CoA mutase (EC 5.4.99.2) involved in propionate oxidation.[5] The *b*-, *d*-, and *e*-propionamide side chains of the corrin ring have been reported to be important for the formation of the apomethionine synthase–Cbl complex.[6] Although there is little information concerning corrinoid specificity of methylmalonyl-CoA mutase, the propionamide side chains of Cbl are assumed to be important in the binding of Cbl to the enzyme.

Euglena gracilis z, a protozoan, has been reported to accumulate CN-Cbl(*b*-OH), (*d*-OH) and (*e*-OH) and to synthesize complete Cbl from the CN-Cbl acid derivatives,[7] suggesting the occurrence of the enzyme involved in the conversion of each of the acid derivatives to CN-Cbl (CN-Cbl monocarboxylic acid amide synthase) in this organism. There is little information available on whether mammalian cells contain the complete Cbl synthetic system found in *E. gracilis* z and how much the side chains of Cbl are involved in physiological roles and nutritional effects of Cbl in mammalian cells.

In this paper, we describe the effects of Cbl analogues with differences in the side chains on Cbl-deficiency and also discuss the involvement of the side chains in the physiological roles of Cbl.

Materials and Methods

Animals and diets. Fifty-six male weanling Wistar rats (3 wk old, 40 ± 5 g), born to 20-wk-old parents fed a Cbl-deprived diet, were used in all the studies. Ten-wk-old parent rats were obtained from Clea Inc. (Tokyo, Japan).

The Cbl-deprived diet was prepared as described previously[8] and used in the experiments. The 3-wk-old weanling rats were housed in individual metabolic cages at 24°C in a room with a 12-h light : dark cycle. They were fed the Cbl-deprived diet *ad libitum* for 11 wk more and had free access to tap water.

Preparation of Cbl analogues. CN-Cbl was obtained from Sigma Co., St. Louis, MO. CN-Cbl(*b*-OH), CN-Cbl(*d*-OH), and CN-Cbl(*e*-OH) were prepared as described by Toraya *et al.*[4] CN-Cbl(*c*-lactam) was prepared by the method of Bonnett *et al.*[9] These Cbl analogues were further purified by HPLC and their purity was checked as described previously.[7]

The revised structural assignments[10,11] of the CN-Cbl(*b*-OH), CN-Cbl(*d*-OH), and CN-Cbl(*e*-OH) were used in the experiments because the assignments of the acid derivatives used in the experiments before 1980 were incorrect.

Cbl analogue feeding experiments. The Cbl-deficient 14-wk-old rats were fed diets supplemented with CN-Cbl or its analogues (25 μg/kg diet) *ad libitum*, replacing the Cbl-deprived diet. The supplemented diet furnished each rat ~500 ng Cbl/d, as calculated from the amount of the food consumed daily (~20 g diet per day per rat). All rats had free access to water. Rats were fed for 5 wk.

† To whom correspondence should be addressed.

Abbreviations: Cbl, vitamin B_{12} or cobalamin; CN-Cbl, cyanocobalamin; AdoCbl, 5'-deoxyadenosylcobalamin; CN-Cbl(*b*-OH), cyanocobalamin-*b*-monocarboxylate; MeCbl, methylcobalamin; CN-Cbl(*d*-OH), cyanocobalamin-*b*-monocarboxylate; CN-Cbl(*e*-OH), cyanocobalamin-*b*-monocarboxylate; CN-Cbl(13-epi), cyano-13-epicobalamin; CN-Cbl(*c*-lactam), cyanocobalamin(*c*-lactam); MMA, methylmalonate, IF, intrinsic factor; TC, transcobalamin II.

In the case of intravenous feeding of the Cbl analogues, the Cbl-deficient rats were injected intravenously with 500 ng of each Cbl analogue every 24 h for 7 d.

Assays. Urinary MMA was assayed by the method of Giorgio and Plaut.[12] Extraction of Cbl from the liver homogenate was done with boiling with KCN at an acid pH.[13] Cbl was assayed with a commercially available Cbl-assay kit (radiodilution method using hog intrinsic factor, Corning Medical, Medfield, MA).

Statistics. Statistical significance was analyzed using Student's *t*-test; $p<0.05$ was considered significant. All values are presented as means ± SD.

Results

Cbl analogues with alternations in the side chains of the corrin ring were synthesized chemically to study the effects of the Cbl analogues on Cbl deficient status in rats. The structural formulae of Cbl and partial structures of the Cbl analogues used in this study are shown in Fig. 1.

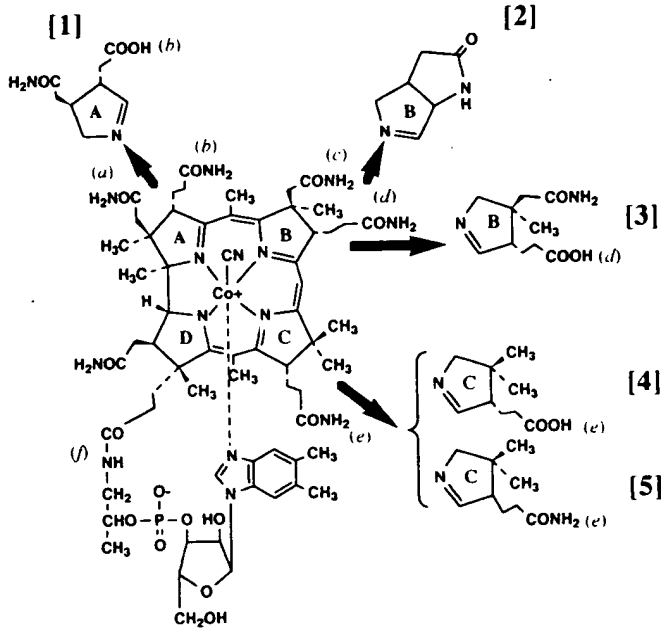


Fig. 1. Structural Formulae of Cbl and Partial Structures of Cbl Analogues.

The partial structures of analogues show only those portions of the molecule that differ from CN-Cbl. 1, CN-Cbl(*b*-OH); 2, CN-Cbl(*c*-lactam); 3, CN-Cbl(*d*-OH); 4, CN-Cbl(*e*-OH); 5, CN-Cbl(13-epi).

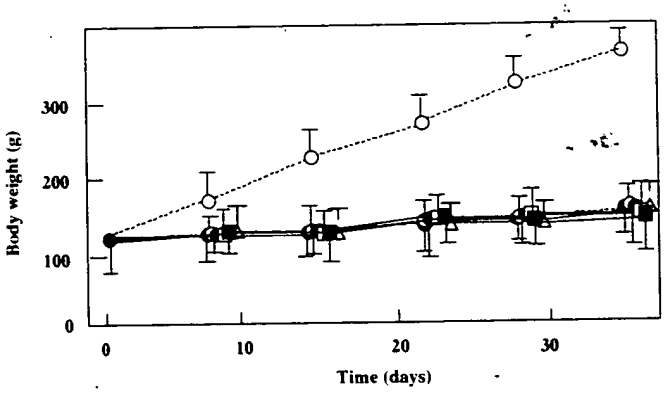


Fig. 2. Effects of Feeding Cbl-analogue-supplemented Diets on Body Weight in the Cbl-deficient Rats.

Body weight of the Cbl-deficient rats with CN-Cbl (---○---); CN-Cbl(*b*-OH) (—○—); CN-Cbl(*d*-OH) (—●—); CN-Cbl(*e*-OH) (—□—); CN-Cbl(*c*-lactam) (—■—); CN-Cbl(13-epi) (—△—); and without CN-Cbl (---●---) feeding. Data represent mean ± SD; $n=4$ rats/group.

Body weights of the 14-wk-old Cbl-deficient rats were ~35% of those of the control rats, that ate a diet including 500 ng of CN-Cbl per kg diet. The Cbl-deficient rats excreted 223.8 ± 136.2 μmol MMA/d in urine and had ~97% lower hepatic Cbl content (1.2 ± 0.7 ng/g tissue). These results indicate that the rats fed the Cbl-deprived diet in our experiments developed a severe Cbl deficiency.

Figure 2 shows the effects of feeding the CN-Cbl analogue-supplemented (~500 ng/d) diets on body weight in the 14-wk-old Cbl-deficient rats. The Cbl-deficient rats recovered ~90% of the body weight of the control rats after 5 wk of CN-Cbl supplementation, but all the Cbl analogues did not affect the weight of the deficient rats. Although urinary MMA excretion became undetectable (< 1 μmol MMA/d) 1 wk after the feeding of the CN-Cbl supplemented diet, the rats fed the CN-Cbl-analogue-supplemented diets continuously excreted substantial amounts of MMA (218.6 ± 112.3 μmol MMA/d) in urine (Fig. 3).

Cbl content was assayed in the livers of the Cbl-deficient rats fed Cbl analogue-supplemented diets for 5 wk (Table). The livers of CN-Cbl-supplemented rats (control) contained substantial amounts of Cbl (72.4 ± 19.1 ng/g tissue). Although CN-Cbl(13-epi)-supplemented rats showed about 80% of liver Cbl content relative to controls, liver Cbl contents of other Cbl analogue-supplemented rats were at levels similar to those of the Cbl-deficient rats. The results indicate that CN-Cbl(13-epi) is effectively absorbed and

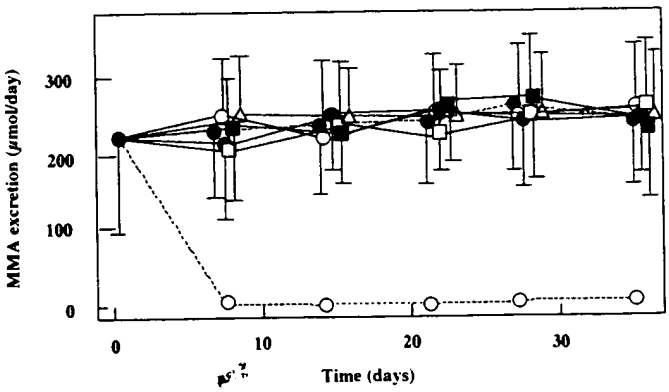


Fig. 3. Effects of Feeding Cbl-analogue-supplemented Diets on Urinary MMA Excretion in the Cbl-deficient Rats.

Urinary MMA excretion of the Cbl-deficient rats with CN-Cbl (---○---); CN-Cbl(*b*-OH) (—○—); CN-Cbl(*d*-OH) (—●—); CN-Cbl(*e*-OH) (—□—); CN-Cbl(*c*-lactam) (—■—); CN-Cbl(13-epi) (—△—); and without CN-Cbl (---●---) feeding. Data represent mean ± SD; $n=4$ rats/group.

Table Hepatic Cbl Concentration in Cbl-deficient Rats Fed Cbl Analogue-supplemented Diets

Compounds	Hepatic Cbl concentration (ng/g tissue)
Cbl-deficient rats	1.2 ± 0.8
CN-Cbl	72.4 ± 19.1*
CN-Cbl (*b*-OH)	3.7 ± 2.9*
CN-Cbl (*d*-OH)	1.2 ± 0.3
CN-Cbl (*e*-OH)	5.9 ± 2.9*
CN-Cbl (13-epi)	57.9 ± 8.9*
CN-Cbl (*c*-lactam)	0.8 ± 0.1

Values are mean ± SD; $n=4$ rats/group. *Significantly different than the Cbl-deficient rat group, $p<0.05$.

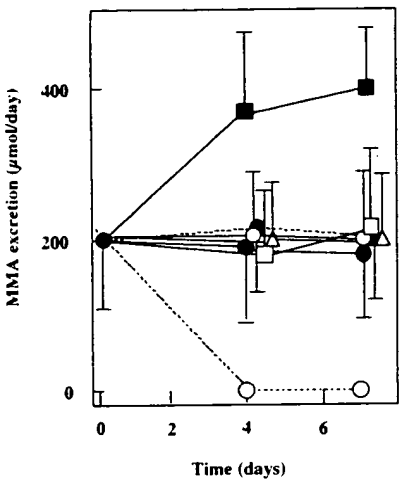


Fig. 4. Effects of Intravenous Feeding of Cbl-analogues on Urinary MMA Excretion in the Cbl-deficient Rats.

Urinary MMA excretion of the Cbl-deficient rats with CN-Cbl (---○---); CN-Cbl(*b*-OH) (—○—); CN-Cbl(*d*-OH) (—●—); CN-Cbl(*e*-OH) (—□—); CN-Cbl(*c*-lactam) (—■—); CN-Cbl(13-epi) (—△—); and without CN-Cbl (---●---) feeding. Data represent mean ± SD; $n=4$ rats/group.

accumulated in the Cbl-deficient rats, but it did not support an increase in growth of the Cbl analogue-supplemented rats (Fig. 2).

Figure 4 shows the effects of intravenous feeding of Cbl analogues on MMA excretion in the Cbl-deficient rats. The 14-wk-old Cbl-deficient rats were injected with 500 ng of each of the Cbl analogues every 24 h for 7 d. Urinary MMA excretion became undetectable 4 d after feeding CN-Cbl, but CN-Cbl(*b*-OH), CN-Cbl(*d*-OH), CN-Cbl(*e*-OH), and CN-Cbl(13-epi) did not affect urinary MMA excretion in the Cbl-deficient rats, indicating that these CN-Cbl analogues can not improve the Cbl-deficient status of the rats. In the CN-Cbl(*c*-lactam)-supplemented rats, urinary MMA excretion increased to about twice as much as that in the Cbl-deficient rats after 7 d of feeding. The results indicate that CN-Cbl(*c*-lactam) has the ability to make the rats severe Cbl-deficient status worse.

Discussion

When the 14-wk-old Cbl-deficient rats were fed the CN-Cbl and CN-Cbl analogue-supplemented diets (~500 ng Cbl/d) for 5 wk, the body weight of the CN-Cbl-supplemented rats increased significantly, but those of all the Cbl analogue-supplemented rats did not at all (Fig. 2). Although urinary MMA excretion as an index of Cbl-deficiency became undetectable 1 wk after the feeding of the CN-Cbl-supplemented diet, all the Cbl analogue-supplemented rats continuously excreted substantial amounts of MMA in urine (Fig. 3). These results indicate that Cbl-deficiency of the rats are improved only by the feeding of CN-Cbl, but not by any other analogues.

The *b*-, *d*-, and *e*-propionamide side chains of the corrin ring of Cbl have been reported to be important in the binding of Cbl to IF and TC,[2] both of which are involved in the intestinal absorption and subsequent plasma transport of Cbl.[14] The order of the contribution of these propionamides for the binding of Cbl to IF and TC has been shown to be $d>b>e$.[2] IF and TC bind CN-Cbl(13-epi) with similar affinity for CN-Cbl, but not CN-Cbl(*c*-lactam).[2] The corrinoid specificity of IF or TC suggests that a physiological amount of CN-Cbl(*b*-OH), CN-Cbl(*d*-OH), CN-Cbl(*e*-OH), or CN-Cbl(*c*-lactam) is not absorbed into the body through the intestinal tract. The Table indicates that CN-Cbl(13-epi) is effectively absorbed and accumulated in the Cbl-deficient rats. Since CN-Cbl(13-epi)-supplemented rats continuously excreted substantial amount of MMA in urine, AdoCbl(13-epi) would not function as a coenzyme of methylmalonyl-CoA mutase. It has been reported that CN-Cbl(13-epi) cannot stimulate *Escherichia coli* and *E. gracilis* z growth,[7,15] and that AdoCbl(13-epi) is also inactive on AdoCbl-dependent dioldehydrase.[16]

To bypass the process of the intestinal absorption of Cbl, the Cbl-deficient rats were injected intravenously with 500 ng of the Cbl analogues every 24 h for 5 d. Urinary MMA excretion became undetectable 4 d after the administration of CN-Cbl or CN-Cbl(*b*-OH), but CN-Cbl(*d*-OH), CN-Cbl(*e*-OH), and CN-Cbl(13-epi) did not affect urinary MMA excretion in the Cbl-deficient rats (Fig. 4). *Euglena gracilis* z has been reported to synthesize "complete Cbl" from the acid derivatives,[7] but the results in Fig. 4 suggest that mammalian cells do not contain the complete Cbl synthetic system that was found in *Euglena* cells.

In the rats administered CN-Cbl(*c*-lactam) intravenously for 7 d, urinary MMA excretion increased about twice as much as those of other Cbl analogue-supplemented rats (Fig. 4), suggesting that CN-Cbl(*c*-lactam) can act as a powerful Cbl-antagonist, but CN-Cbl(*b*-OH), CN-Cbl(*d*-OH), CN-Cbl(*e*-OH), and CN-Cbl(13-epi) cannot. Rats treated with CN-Cbl(*c*-lactam) have been reported to develop decreased hepatic Cbl levels,[17] and decreased activities of methylmalonyl-CoA mutase and methionine synthase.[18] The results (Fig. 4) that CN-Cbl(*c*-lactam) has the ability to make the rats worse in Cbl-deficient status supports the above observations; the CN-Cbl(*c*-lactam) treatment is one of the most useful methods for preparation of Cbl-deficient rats.

Regarding the mechanism of development of Cbl-deficiency caused by the treatment with CN-Cbl(*c*-lactam), it has been reported that OH-Cbl(*b*-OH), OH-Cbl(*d*-OH), OH-Cbl(*e*-OH), and OH-Cbl(*c*-lactam) are less effective ligands in the activation of apomethionine synthase than OH-Cbl, but they are capable of fully activating the apoenzyme.[6] The observations suggest that CN-Cbl(*c*-lactam)-induced Cbl-deficiency is not due to the impairment of methionine synthase function. Although there is little information available on corrinoid specificity of methylmalonyl-CoA mutase, treatment of rats with the CN-Cbl(*c*-lactam) would impair the enzyme function and lead to methylmalonic aciduria.

References

1) Z. Schneider and A. Stroinski, in "Comprehensive B12," Walter de Gruyter, Berlin, New York, 1987, pp. 225–266.
2) J. F. Kolhouse and R. H. Allen, *J. Clin. Invest.*, **60**, 1381–1392 (1977).
3) E. Stupperich and E. Nexo, *Eur. J. Biochem.*, **199**, 299–303 (1991).
4) T. Toraya, E. Krodel, A. S. Mildvan, and R. H. Abeles, *Biochemistry*, **18**, 417–426 (1979).
5) J. M. Poston and T. C. Stadman in "Cobalamin," ed. by B. M. Babior, John Wiley & Sons, New York, London, Sydney, Toronto, 1975, pp. 111–212.
6) J. F. Kolhouse, C. Utley, S. P. Stabler, and R. H. Allen, *J. Biol. Chem.*, **266**, 23010–23015 (1991).
7) F. Watanabe, Y. Nakano, and E. Stupperich, *J. Gen. Microbiol.*, **138**, 1807–1813 (1992).

8) F. Watanabe, Y. Nakano, N. Tachikake, H. Saido, Y. Tamura, and H. Yamanaka, *J. Nutr.*, **121**, 1948–1954 (1991).
9) R. Bonnett, J. R. Cannon, U. M. Clark, A. W. Johnson, L. F. J. Parker, E. L. Smith, and A. Todd, *J. Am. Chem. Soc.*, **1957**, 1158–1168.
10) D. L. Anton, H. P. C. Hogenkamp, T. E. Walker, and N. A. Matwiyoff, *J. Am. Chem. Soc.*, **102**, 2215—2219 (1980).
11) T. G. Pagano and L. G. Marzilli, *Biochemistry*, **28**, 7213–7223 (1989).
12) A. J. Giorgio and G. W. E. Plaut, *J. Lab. Clin. Med.*, **66**, 667–676 (1965).
13) E. P. Frenkel, R. Prough, and R. L. Kitchens, *Methods Enzymol.*, **67**, 31–40 (1980).
14) B. Seetharam and D. H. Alpers, *Ann. Rev. Nutr.*, **2**, 343–369 (1982).
15) R. Bonnett, J. M. Godfrey, V. B. Math, E. Edmond, H. Evans, and O. J. R. Hodder, *Nature*, **229**, 473–476 (1971).
16) T. Toraya, T. Shirakashi, S. Fukui, and H. P. C. Hogenkamp, *Biochemistry*, **14**, 3949–3952 (1975).
17) E. P. Brass, A. G. Tahilian, R. H. Allen, and S. P. Stabler, *J. Nutr.*, **120**, 290–297 (1990).
18) S. P. Stabler, P. D. Marcell, E. R. Podell, R. H. Allen, and J. Lindenbaum, *Blood*, **64** (Suppl. 1), 42a (1984).

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Kroes et.
Journal/Book Title:	Cancer Chemotherapy and Pharmacology
Article Title:	
Volume (Issue):	17/2
Pages:	114-120
Year of Publication:	1984
Publisher:	
Remarks:	

FC8035 FR. 10/3/8

3719892

39781

STIC Use Only Accession Number:

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

Cancer Chemother Pharmacol (1986) 17: 114–120

Cancer Chemotherapy and Pharmacology

Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (vitamin B12) by nitrous oxide

A. C. M. Kroes, J. Lindemans, M. Schoester, and J. Abels

Institute of Hematology, Erasmus University, Rotterdam, The Netherlands

Summary Exposure to nitrous oxide interferes selectively with the coenzyme function of vitamin B12 and causes inactivation of methionine synthetase, with subsequent impairment of folate metabolism and reduction of cellular proliferation. In a rat leukemia model (BNML) we investigated the combined administration of nitrous oxide, inactivating vitamin B12, and methotrexate (MTX), a folate antagonist inhibiting the enzyme dihydrofolate reductase. Through different mechanisms, both agents decrease the availability of tetrahydrofolate, and subsequently of other reduced folates, with increased impairment of folate-dependent synthesis of thymidylate. Effects on leukemic growth and on hematological values in rats demonstrated enhancement of the therapeutic effect of MTX by exposure to nitrous oxide. With several treatment schedules, the results of combined treatment were seen to be better than additive when compared with the effects of single agents. In particular, pretreatment of leukemic rats with nitrous oxide for 3 days before administration of MTX appeared effective. With higher doses of MTX, concomitant exposure to nitrous oxide even resulted in toxic effects. These findings were in accordance with the results of some metabolic studies performed in leukemic rats. De novo synthesis of thymidylate in leukemic cells, when studied by means of the deoxyuridine suppression test, showed the most severe disturbance with combined treatment consisting in MTX (0.5 mg/kg) and nitrous oxide pretreatment for 3 days. Intracellular levels of folate and dTTP were lowest with 2 and 3 days' pretreatment before MTX, respectively. It is concluded that this interaction of nitrous oxide and MTX can result in enhanced metabolic and therapeutic effects of low doses of MTX. Inactivation of vitamin B12 appears to be a potentially useful addition in cancer chemotherapy.

Introduction

The similarity of hematological disturbances caused by deficiency of either folic acid or cobalamin (vitamin B12) is well established. Both vitamins are involved in pathways essential in the synthesis of nucleotides, and consequently of DNA. The important function of folic acid in cellular proliferation is also reflected in the striking cytostatic activity of folate antagonists of which methotrexate (MTX) is the best known example. This antimetabolite is widely used in cancer chemotherapy [14]. In contrast, until recently, the role of vitamin B12 in neoplastic growth has remained unclear, because no effective method was available to interfere with its coenzyme function. In 1978, however, it was recognized that selective inactivation of vitamin B12 could be achieved with exposure to the anesthetic gas nitrous oxide, or N_2O [1, 7]. Megaloblastic hematopoiesis after prolonged exposure to nitrous oxide had been observed much earlier [21], and a chemical interaction of nitrous oxide with complexes of cobalt was also known for some time [2]. It appeared that a specific oxidative action of nitrous oxide on the cobalt moiety of vitamin B12 caused a nearly complete inactivation of the methylcobalamin-requiring enzyme methionine synthetase, or 5-methyltetrahydrofolate homocysteine methyltransferase (E. C. 2.1.1.13). Nitrous oxide effectively established a state of functional deficiency of vitamin B12, with severely disturbed folate metabolism [6] and toxic effects on hematopoiesis [38]. Methionine synthetase is essential in folate metabolism, because it provides the only pathway by which 5-methyltetrahydrofolate, the major extracellular folate, can be converted into tetrahydrofolate (THF). THF and other, subsequently formed, reduced folates can be converted into folylpolyglutamates, which are then retained in the cell and are important coenzymes in one-carbon transfer reactions. De novo synthesis of thymidylate requires such folate-dependent methylation. MTX also interferes with folate metabolism by limiting the generation of THF, but through a different mechanism. Its inhibition of dihydrofolate (DHF) reductase prevents the reconversion of DHF to THF.

It has been shown that the effect of nitrous oxide on vitamin B12 can be utilized to reduce growth of leukemia in vitro [18] and in vivo, in rats [20]. This effect is associated with a disturbance of folate-dependent de novo synthesis of thymidylate. Therefore, it appears that nitrous oxide and MTX, through inhibition of different pathways, ultimately may have similar effects on folate metabolism. These observations suggest that the inactivation of vitamin B12 can modify, and possibly enhance, the efficacy of MTX. In vitro studies on human bone marrow have demonstrated a synergistic effect of MTX and nitrous oxide

Offprint requests to: A. C. M. Kroes, Institute of Hematology, Erasmus University Rotterdam, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands

Abbreviations used in this paper: MTX, methotrexate; THF, tetrahydrofolate; DHF, dihydrofolate; dTTP, deoxythymidine triphosphate.

with regard to impairment of nucleotide synthesis [17]. The purpose of the present study is to investigate the influence of nitrous oxide on the effects of MTX in vivo, using a rat leukemia model: the Brown Norway myeloid leukemia (BNML). This transplantable acute promyelocytic leukemia has been described in detail elsewhere [12] and is considered to be a suitable model for chemotherapeutic studies [40]. In addition to experiments intended to assess effects on leukemic growth, a number of metabolic studies were performed in leukemic rats, to investigate some effects of treatment on folate metabolism. These studies included deoxyuridine suppression tests, and determinations of intracellular folate and deoxythymidine triphosphate (dTTP) levels.

Materials and methods

Animals. Male rats of the Brown Norway inbred strain were used at the age of 12–16 weeks (body weight 200–275 g). Food and water were supplied ad libitum during the experiments.

Brown Norway myeloid leukemia (BNML). Cryopreserved leukemic cells were kindly provided by Dr. A. Hagenbeek from the Radiobiological Institute (TNO), Rijswijk, The Netherlands, where this transplantable rat leukemia model was developed. Origin, classification and proliferation kinetics were described elsewhere [12]. For leukemia transfer in experimental series, spleen cells of fully leukemic animals were used. A standard dose of 10^7 cells suspended in Hanks' balanced salt solution was injected IV, which leads to progressive leukemic infiltration of bone marrow, spleen and liver, with death after 20–24 days. Spleen and liver weights, steadily increasing in the course of leukemia, are reliable indicators of tumor load and, along with hematological determinations, can be used effectively to assess effects of chemotherapy [12]. To avoid a gradual change in growth properties, serial transplantations were limited to only two passages, after which spleen cells were used from rats freshly inoculated with cells from a cryopreserved stock.

Treatment with nitrous oxide and MTX. Leukemic rats were treated according to different schedules in groups of at least four. Exposure to nitrous oxide was carried out in a 40-l flow chamber into which a mixture of 50% nitrous oxide and 50% oxygen was blown at a rate of 500 ml/min. Oxygen concentration was monitored with an oxygen analyzer (Teledyne Analytical Instruments). Carbon dioxide, water, and contaminating volatile compounds were eliminated in a cleaning circuit, essentially as described by Rupreht and Dzoljic [37]. Rats not exposed to nitrous oxide were kept in air, but otherwise treated identically. Sodium methotrexate (Ledertrexate SP, from Lederle) was injected IP. Rats not receiving MTX received injections of 0.15 *M* NaCl IP instead.

Evaluation of leukemic growth. Experiments intended to assess effects on leukemic growth were all evaluated by the same procedure. To allow a simultaneous investigation of several aspects of leukemia these experiments were terminated after a fixed period of 18 days (in some instances: 19 days) of leukemia, just before death from leukemia was to be expected. Rats were killed by exsanguination, after recording of body weights. Liver and spleen were carefully removed and weighed. Leukocytes were counted electronically, and in some experiments differential blood cell counts were done. Plasma levels of vitamin B12 were measured in a competitive radioisotope binding assay using purified intrinsic factor [22]. Normal values for organ weights, leukocyte counts and plasma vitamin B12 were derived from at least 12 comparable nonleukemic Brown Norway rats.

Metabolic studies. In separate experiments, rats with advanced leukemia were treated for short periods, after which leukemic cells were used in metabolic studies. Three similar experiments were carried out separately. In each, eight leukemic rats inoculated at day 0 were divided in four pairs. These pairs were treated with nitrous oxide for 3, 2, 1, or 0 days. Immediately afterwards one rat in each pair received MTX, 0.5 mg/kg IP, the other rat receiving saline only. At 18 h after administration of MTX, on day 15 of leukemia, leukemic cells were obtained from the spleens of all rats, washed, and resuspended in Hanks' balanced salt solution. These cell suspensions were counted electronically and used in deoxyuridine suppression tests and determinations of intracellular folate and dTTP.

Deoxyuridine suppression test. This test demonstrates impaired de novo synthesis of thymidylate. ^{3}H-Thymidine incorporation into DNA is measured with and without addition of deoxyuridine. Deoxyuridine will suppress incorporation of ^{3}H-thymidine in DNA if it can be converted to thymidylate through folate-dependent methylation. This suppression is reduced by vitamin B12 or folate deficiency [42], inactivation of vitamin B12 by nitrous oxide [28], and treatment with other agents interfering with de novo synthesis of thymidylate [5].

Leukemic spleen cells (approx. 5×10^6 per test) were used from rats of various groups, as described above. The test was carried out essentially according to Metz [30], with some modification as described elsewhere [20]. Deoxyuridine (Sigma, St. Louis, USA) was used in a concentration of 0.1 mmol/l. All incubations were performed in triplicate. Incorporation of ^{3}H-thymidine (0.3 µCi per test, specific activity 25 Ci/mmol, from Amersham, UK) is expressed as a percentage of the maximal incorporation, measured in each case in incubations without addition of deoxyuridine.

Intracellular folate. In suspensions of leukemic spleen cells, intracellular folate content was determined. After centrifugation a pellet of approx. 10^8 cells was resuspended in a total volume of 1 ml 10% (= 1.3 mol/l) mercaptoethanol, heated in a water bath of 100 °C for 5 min, and cooled. Hog kidney polyglutamate hydrolase, prepared as described by McMartin et al. [29], was added and allowed to incubate at room temperature for 2 h. The samples were frozen at −20 °C until assayed. After thawing, the extracts were centrifugated (1500 *g* for 10 min at 4 °C), and aliquots of the supernatants were used in a folate radioisotope dilution assay, essentially as described by Dunn and Foster [11], with ^{125}I-folic acid (Becton Dickinson, Orangeburg, N.Y., USA) as a tracer, and β-lactoglobulin (Sigma, St. Louis, USA) as a folate binder. 5-Methyl-THF was used as a standard, and results are expressed as picomoles of folate per 10^6 cells.

Intracellular dTTP. In leukemic cells obtained from rats as described above, dTTP was determined using the DNA polymerase assay system originally developed by Solter and Handschumacher [39], with the modifications and corrections published by Hunting and Henderson [13]. A different extraction method was used, however. After being washed once in Hanks' balanced salt solution, cell suspensions were centrifuged and the supernatant was removed. To the pellet of about 10^8 cells, 1.2 µg cyano [^{57}Co]cobalamin, or 10^5 dpm, (Amersham, UK) was added, as an internal standard for cell quantities in the assay. The pellet was then extracted with 5 ml ice-cold 60% methanol and stored at −20 °C until assayed. After centrifugation the supernatant was dried using a rotary evaporator at 25 °C, dissolved in 50 m*M* potassium cacodylate and subsequently used in the DNA polymerase catalyzed assay. DNA polymerase I from *E. coli*, dATP, dTTP, and poly(d(A,T)) were all obtained from Boehringer Mannheim (FRG) and ^{3}H-dATP and ^{3}H-dTTP were from Amersham (UK). Procedures, and calculated corrections for dilution of specific activities by the endogenous nucleotides were carried out as described elsewhere [13]. Concentrations of dTTP are expressed as picomoles per 10^6 cells, based upon counts of the internal standard and cell concentration of the initial suspension.

Results

Effects of growth of leukemia in rats

In Table 1, results of six experiments with several different treatment schedules are summarized. In all these experiments, one group of leukemic rats treated with MTX only is compared with one or more groups of rats treated with a combination of MTX and exposure to nitrous oxide. Most experiments included a group receiving nitrous oxide continuously throughout the treatment period (day 7 to day 18 or 19 of leukemia). In other groups, nitrous oxide treatment was limited to either 3 days before administration of MTX (pretreatment) or 3 days after administration of MTX (post-treatment). This is illustrated in Fig. 1, which shows the treatment schedules as applied in experiment 3 of Table 1. In addition, all experiments included rats receiving no treatment or nitrous oxide only (days 7–19), without MTX. Cumulative results in these rats are also shown in Table 1. From the data in Table 1, it follows that low doses of MTX alone do not have substantial effects on leukemic growth. In all experiments the addition of nitrous oxide enhanced the therapeutic effects of MTX. The differences between rats treated with MTX only and rats treated with both MTX and nitrous oxide (continuously) are statistically significant according to Wilcoxon's nonparametric rank sum test. For all three parameters of leukemia, results did not overlap, yielding p-values of 0.05 or less, in any of the experiments 1–4. In addition, the results of combined treatment appeared to be better than additive in these experiments, with the possible exception of experiment 1, in which the lowest dose of MTX was administered. In other cases, the reduction of leukemic growth obtained with combined treatment is often greater than the added effects of the two single agents, which is indicative of a synergistic interaction. From experiments 3 and 5 it can be derived that by far the major part of the added effect is contributed by the period of exposure to nitrous oxide before administration of MTX (pretreatment schedules). In experiment 6 the highest dose of MTX was used (4 mg/kg). In this experiment, rats treated with both MTX and nitrous oxide (continuously) died prematurely at 14–16 days after inoculation, without any evidence of leukemia. Spleen and liver weights at autopsy were subnor-

Table 1. Effects of treatment on growth of leukemia

Expt no	Treatment	No. of. rats	Spleen weight[a] (g) mean ± SEM	Liver weight[b] (g) mean ± SEM	Leukocytes[c] (10^9) mean ± SEM
	None (untreated controls)	22	3.90 ± 0.09	17.39 ± 0.45	24.8 ± 2.1
	N_2O, continuous (days 7–18/19)	14	2.89 ± 0.09	14.33 ± 0.44	11.1 ± 1.0
1	MTX, 1 × 0.5 mg/kg (day 11)	5	3.64 ± 0.10	15.60 ± 0.69	18.1 ± 1.2
	+ N_2O, continuous (days 7–19)	5	2.34 ± 0.10	12.50 ± 0.55	7.7 ± 0.7
2	MTX, 2 × 0.5 mg/kg (days 10 and 14)	4	3.29 ± 0.09	12.95 ± 0.52	16.6 ± 1.3
	+ N_2O, continuous (days 7–18)	4	1.43 ± 0.14	9.76 ± 0.47	3.8 ± 0.5
3	MTX, 2 × 0.5 mg/kg (days 10 and 16)	4	3.38 ± 0.18	16.04 ± 0.78	20.2 ± 1.4
	+ N_2O, 2 × 3 days after MTX (days 10–13/16–19)	4[d]	3.32	17.05	20.0
	+ N_2O, 2 × 3 days before MTX (days 7–10/13–16)	4	2.37 ± 0.11	13.08 ± 0.44	8.1 ± 0.6
	+ N_2O, continuous (days 7–19)	4	1.97 ± 0.17	11.68 ± 0.81	7.0 ± 1.1
4	MTX, 1 × 2 mg/kg (day 10)	4	3.44 ± 0.14	14.00 ± 0.46	20.8 ± 2.9
	+ N_2O, 1 × 3 days before MTX (days 7–10)	4	2.58 ± 0.24	12.59 ± 0.94	7.7 ± 1.0
	+ N_2O, continuous (days 7–19)	4	1.95 ± 0.08	11.47 ± 0.17	6.1 ± 0.6
5	MTX, 2 × 1 mg/kg (days 6 and 14)	4	3.19 ± 0.22	12.37 ± 0.59	15.9 ± 1.8
	+ N_2O, 2 × 3 days after MTX (days 6–9/14–17)	4	3.37 ± 0.10	14.70 ± 0.80	14.0 ± 0.7
	+ N_2O, 2 × 3 days before MTX (days 3–6/11–14)	4	2.31 ± 0.14	10.64 ± 0.51	10.8 ± 1.2
6	MTX, 1 × 4 mg/kg (day 10)	4	2.85 ± 0.32	13.30 ± 0.74	14.9 ± 1.1
	+ N_2O, 1 × 3 days before MTX (days 7–10)	4	1.47 ± 0.43	10.12 ± 0.49	4.2 ± 1.4
	+ N_2O, continuous (from day 7)	4	all rats died (without evidence of leukemia)		

[a] Normal spleen weight in comparable nonleukemic BN rats: 0.45 ± 0.07 g
[b] Normal liver weight in comparable nonleukemic BN rats: 8.25 ± 0.99 g
[c] Normal value of leukocyte count in nonleukemic BN rats: 3.9 ± 0.4 × 10^9/l
[d] 2 rats dying prematurely of leukemia are not included

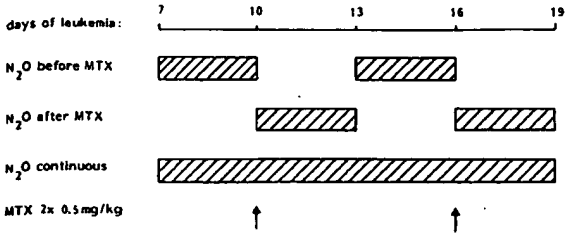


Fig. 1. Treatment of leukemic rats with nitrous oxide (N_2O) and methotrexate (MTX), as given in experiment 3 of Table 1. Periods of exposure to nitrous oxide are *shaded*, and *arrows* indicate time of administration of MTX. Rats receiving MTX or nitrous oxide only and untreated rats were also included

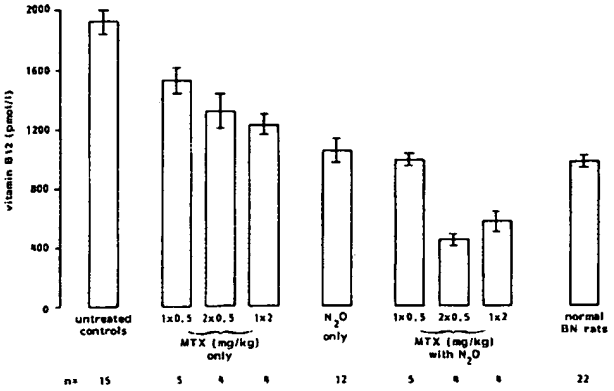


Fig. 2. Plasma levels of vitamin B12 in leukemic rats treated in several experiments with different doses of MTX. Groups with combined treatment received nitrous oxide continuously (days (7–18/19 of leukemia). Values in normal (nonleukemic) BN rats are also shown. *Bars* indicate SEM

mal, and body weight was seriously reduced (77% of weight before treatment). This was considered to be a toxic effect of treatment. In other experiments, however, no toxicity was observed and nitrous oxide treatment was well tolerated by rats without evident effects on consciousness. Even with combined treatment, loss of body weight was always less than 10%.

In some experiments differential blood cell counts were made, which demonstrated that the observed reductions in peripheral leukocyte counts were also accompanied by a striking relative decrease of leukemic cells (promyelocytes), as shown in Table 2.

Plasma levels of vitamin B12, as determined in these experiments, are presented in Fig. 2. Compared with normal BN rats, vitamin B12 levels in untreated leukemic controls are very high. With treatment these levels are reduced, as is shown for three doses of MTX. Exposure to nitrous oxide alone has marked effects on the vitamin B12 level in plasma, but much lower and even subnormal levels are found after combined treatment.

Metabolic effects

In separate experiments, pairs of leukemic rats were treated with nitrous oxide for 0, 1, 2, or 3 days. One rat of each pair subsequently received MTX (0.5 mg/kg), and 18 h afterwards leukemic cells of all rats were used for deoxyuri-

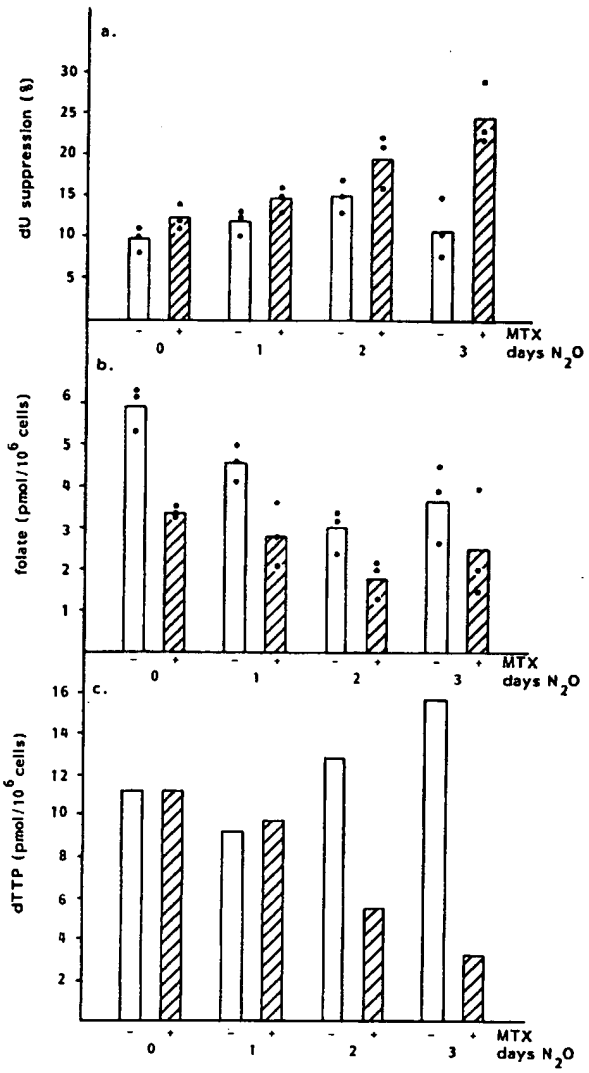


Fig. 3a–c. Results of metabolic experiments with leukemic spleen cells after in vivo treatment of rats with 0–3 days' exposure to nitrous oxide, followed or not by administration of MTX (0.5 mg/kg): **a** deoxyuridine suppression values, expressed as percentages of maximal incorporation of ^{3}H-thymidine, in incubations without deoxyuridine; **b** intracellular levels of folate (pmol/10^6 cells); **c** intracellular levels of dTTP (pmol/10^6 cells). *Shaded columns* indicate MTX treatment. The experiments were performed 18 h after administration of MTX and/or exposure to nitrous oxide

dine suppression tests and determinations of intracellular folate and dTTP. This experimental procedure was repeated twice, and results of all rats used in this study are presented together (Fig. 3).

Deoxyuridine suppression tests (Fig. 3a) show increased disturbance with longer duration of nitrous oxide exposure before the administration of MTX. Higher values in this test indicate a decreased ability of deoxyuridine to suppress the uptake of ^{3}H-thymidine, demonstrating impaired de novo synthesis of thymidylate. MTX without nitrous oxide treatment is clearly less effective: mean values are 12% without nitrous oxide and 25% with 3 days of pretreatment. Figure 3b shows the results of intracellular folate determinations. Both nitrous oxide and MTX de-

Table 2. Effect of treatment on differential blood cell counts

Treatment	No. of. rats	Promyelocytes[a] (leukemic cells)	Lymphocytes[a]	Neutrophils[a]
None (untreated controls)	8	17.4 ± 1.9	74.4 ± 2.0	8.3 ± 1.6
N_2O, continuous (days 7 – 18/19)	6	13.5 ± 3.4	83.2 ± 2.7	3.3 ± 0.8
MTX, 2 × 0.5 mg/kg (days 10 and 14)	4	13.5 ± 2.2	74.5 ± 1.8	12.0 ± 1.0
+ N_2O, continuous (days 7 – 18)	4	0.8 ± 0.2	98.3 ± 0.6	1.0 ± 0.4
MTX, 1 × 2 mg/kg (day 10)	3	30.6 ± 1.5	65.0 ± 1.5	4.3 ± 1.2
+ N_2O, 1 × 3 days before MTX (days 7 – 10)	3	8.0 ± 6.0	90.3 ± 6.2	1.7 ± 0.3
+ N_2O, continuous (days 7 – 19)	4	3.3 ± 0.9	95.5 ± 1.0	1.3 ± 0.2
MTX, 1 × 4 mg/kg (day 10)	4	13.3 ± 3.9	81.5 ± 5.2	5.3 ± 1.6
+ N_2O, 1 × 3 days before MTX (days 7 – 10)	4	2.0 ± 0.7	97.5 ± 0.9	0.5 ± 0.3
Normal BN rats (nonleukemic)	5	0	91.4 ± 1.4	6.0 ± 1.5[b]

[a] Expressed as percentages of the total number of nucleated cells, counting 200 cells, with indication of SEM
[b] With additional 0.4% eosinophils and 2.2% monocytes

creased folate levels, and the lowest levels are found with combined treatment after 2 days of nitrous oxide exposure. With 3 days of nitrous oxide exposure it appears that folate contents recover to some extent. Figure 3c, presenting levels of intracellular dTTP, is based upon the last metabolic experiment only, involving eight rats, in contrast to the other results. From this experiment it can be concluded that dTTP levels in leukemic cells are lowest with combined treatment consisting in MTX and 2 or 3 days' pretreatment with nitrous oxide. Nitrous oxide treatment alone appears to cause increased levels of dTTP, but it should be emphasized that these and other values were obtained 18 h after exposure and some recovery may have occurred.

Discussion

Nitrous oxide interferes specifically with the coenzyme function of vitamin B12 and thereby inactivates methionine synthetase [8]. This severely affects folate metabolism, because methionine synthetase is required in the conversion of 5-methyl-THF, the predominant extracellular folate, into THF. This conversion is essential for folate coenzyme functions and also for the cellular retention of folates. In contrast to other reduced folates, 5-methyl-THF is not a substrate for synthesis of folylpolyglutamates, as is evident from metabolic studies [23, 27] and from properties of the purified enzyme [31]. The synthesis of folylpolyglutamates is decreased by nitrous oxide [27, 34, 35], which explains the serious cellular depletion of folates occurring on exposure [25]. The decreased availability of reduced folates impairs folate-dependent synthesis of thymidylate, and subsequently reduces DNA synthesis and cellular proliferation. The inhibition of leukemic growth by nitrous oxide in vitro has been demonstrated [18], and in a previous study we described in vivo antileukemic effects of nitrous oxide in rats [20]. We also showed that these effects were enhanced in combined treatment with cycloleucine, which inhibits the conversion of methionine into S-adenosylmethionine and indirectly interferes with folate metaboilism [19]. A number of studies have investigated the combination of nitrous oxide, as a vitamin B12-inactivating agent, and MTX, as a typical folate antagonist. Kano et al. [17] have demonstrated synergistic effects with regard to inhibition of thymidylate synthesis in normal human bone marrow. Black and Tephly [4] compared metabolic effects of both agents in rat liver cells. The inhibition of methionine synthetase by nitrous oxide considerably decreased the availability of THF while the inhibition of DHF reductase by MTX was much less effective. This difference probably can be explained by the low activity of thymidylate synthetase in liver cells. Dudman et al. [10] found increased sensitivity of leukemic cell lines to MTX with nitrous oxide-induced inhibition of methionine synthetase, which was further exploited by the use of 5-methyl-THF instead of 5-formyl-THF as a rescue agent.

The present study shows effects of combined therapy with nitrous oxide and MTX on in vivo growth and metabolism of rat leukemia. The exposure of rats to nitrous oxide enhanced inhibition of leukemic growth by low doses of MTX. A period of exposure before the administration of MTX (pretreatment) appeared essential for this effect. The effects on leukemic infiltration in spleen and liver correlated well with hematologic results (total and differential leukocyte counts) and with determinations of vitamin B12 in plasma. An interesting feature of this leukemia is a continuous rise of vitamin B12 levels in the course of leukemic growth [20], which is also observed in human acute promyelocytic leukemia [36]. Plasma vitamin B12 can be used as a kind of tumor marker, and treatment leads to reduced levels. The particularly striking decrease caused by nitrous oxide, however, is explained by its specific effect on cobalamin. Analogues of cobalamin are formed after oxidation and are excreted rapidly [33]. In addition to effects on leukemic growth, some metabolic aspects of this interaction were studied in leukemic cells after in vivo treatment of rats. On the basis of the results discussed before, pretreatment with nitrous oxide was administered for periods up to 3 days. Deoxyuridine suppression tests demonstrated increased disturbance of de novo synthesis of thymidylate with longer duration of nitrous oxide pretreatment before MTX. Treatment with a single agent was clearly less effective. Simultaneous determination of intracellular folates, to confirm the presumed cellular folate depletion, indeed showed a decrease in folate content, particularly with combined treatment. Some comments on these folate measurements are warranted however. In the radioisotope competitive binding assay the

β-lactoglobulin folate binder is used, and probably not all relevant folate derivatives show the same affinity for this binder. Our own observations (not included in this study) indicated that 5-methyl-THF has slightly lower affinity than THF. As these folates are intracellularly predominant [4, 29], the observed decrease in total folate content could also be explained, at least partially, by a shift of folates from THF toward 5-methyl-THF. This, however, is functionally about equivalent, because the conversion of 5-methyl-THF is blocked on nitrous oxide exposure, as discussed before. Moreover, the results of this folate radioassay in the measurement of tissue folates were recently found to be comparable to microbiological assays [26]. Changes in cellular folate on nitrous oxide exposure in our study are similar to earlier observations [25, 35], including an indication of recovery at more than 2 days of exposure, an adaptive mechanism which is not yet understood. Finally, decreased dTTP levels show that the impairment of folate-dependent synthesis of thymidylate has noticeable effects on this direct precursor of DNA. Considered together, the findings in metabolic experiments are in accordance with the results of studies directed at inhibition of growth, showing maximum effects after about 3 days of pretreatment with nitrous oxide before MTX.

Several potential mechanisms could explain the results of this interaction. First, a reduction of intracellular folates by nitrous oxide pretreatment can obviously induce greater susceptibility to folate antagonists [16]. A second mechanism is closely related and concerns the reduced synthesis of folylpolyglutamates on nitrous oxide exposure. Polyglutamation of MTX, leading to increased activity and cellular retention [15], occurs in competition with normal folate substrates [3, 16]. In cells pretreated with nitrous oxide a larger proportion of MTX may be converted into polyglutamate forms. A third potential mechanism to explain the results relates to the observed marked increase in activity of thymidylate synthetase, after nitrous oxide exposure of up to 3 days [9]. It is well established that thymidylate synthetase has a central role in mediating the cytotoxic effects of MTX [32, 41], because this enzyme actually causes THF depletion by its conversion of reduced folates into DHF. Increased activity of this enzyme, as a possible adaptation to nitrous oxide treatment, thus may result in enhanced effects of MTX. All these mechanisms can explain the observed importance of pretreatment with nitrous oxide before MTX.

The results presented in this study demonstrate that in vivo metabolic manipulation of leukemic cells with nitrous oxide can enhance metabolic and therapeutic effects of MTX. The clinical relevance of this interaction is illustrated by the recent observation of increased toxicity of adjuvant chemotherapy involving MTX started directly postoperatively [24], which the authors attributed to inactivation of vitamin B12 by nitrous oxide during anesthesia. Our findings lend support to this suggestion. In experimental chemotherapy, the inactivation of vitamin B12 represents a new method to enhance activity of MTX. The relative contributions of increased polyglutamation of MTX, and increased activity of thymidylate synthetase, should be subjects of further research. It also remains to be demonstrated that the effects described are applicable to human leukemia, but it is known that man is more susceptible to vitamin B12 deficiency than any animal [6]. Finally, these results indicate the significance of vitamin B12 in leukemic proliferation and the value of vitamin B12-related metabolism as an additional target in cancer chemotherapy.

References

1. Amess JAL, Burman JF, Ress GM, Nancekievill DG, Mollin DL (1978 Megaloblastic haemopoiesis in patients receiving nitrous oxide. Lancet 2: 339
2. Banks RGS, Henderson RJ, Pratt JM (1968) Reactions of gases in solution: III. Some reactions of nitrous oxide with transition-metal complexes. J Chem Soc [A] 12: 2886
3. Barford PA, Blair JA, Malghani MAK (1980) The effect of methotrexate on folate metabolism in the rat. Br J Cancer 41: 816
4. Black KA, Tephly TR (1983) Effects of nitrous oxide and methotrexate administration on hepatic methionine synthetase and dihydrofolate reductase activities, hepatic folates, and formate oxidation in rats. Mol Pharmacol 23: 724
5. Bruckner HW, Schreiber C, Waxman S (1975) Interaction of chemotherapeutic agents with methotrexate and 5-fluorouracil and its effect on de novo DNA synthesis. Cancer Res 35: 801
6. Chanarin I (1982) The effects of nitrous oxide on cobalamins, folates, and on related events. CRC Crit Rev Toxicol 10: 179
7. Deacon R, Lumb M, Perry J, Chanarin I, Minty B, Halsey MJ, Nunn JF (1978) Selective inactivation of vitamin B12 in rats by nitrous oxide. Lancet 2: 1023
8. Deacon R, Lumb M, Perry J, Chanarin I, Minty B, Halsey M, Nunn J (1980) Inactivation of methionine synthetase by nitrous oxide. Eur J Biochem 104: 419
9. Deacon R, Perry J, Lumb M, Chanarin I (1981) The effect of nitrous oxide-induced inactivation of vitamin B12 on thymidylate synthetase activity of rat bone marrow cells. Biochem Biophys Res Commun 102: 215
10. Dudman NPB, Slowiaczek P, Tattersall MHN (1982) Methotrexate rescue by 5-methyltetrahydrofolate or 5-formyltetrahydrofolate in lymphoblast cell lines. Cancer Res 42: 502
11. Dunn RT, Foster LB (1973) Radioassay of serum folate. Clin Chem 19: 1101
12. Hagenbeek A, Van Bekkum DW (eds) (1977) Proceedings of a workshop on comparative evaluation of the L5222 and the BNML rat leukaemia models and their relevance for human acute leukaemia. Leukemia Res 1: 75
13. Hunting D, Henderson JF (1981) Determination of deoxyribonucleoside triphosphates using DNA polymerase: a critical evaluation. Can J Biochem 59: 723
14. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA (1983) The pharmacology and clinical use of methotrexate. N Engl J Med 309: 1094
15. Jolivet J, Schilsky RL, Bailey BD, Drake JC, Chabner BA (1982) Synthesis, retention and biological activity of methotrexate polyglutamates in cultured human breast cancer cells. J Clin Invest 70: 351
16. Kamen BA, Nylen PA, Camitta BM, Bertino JR (1981) Methotrexate accumulation and folate depletion in cells as a possible mechanism of chronic toxicity to the drug. Br J Haematol 49: 355
17. Kano Y, Sakamoto S, Sakuraya K, Kubota T, Hida K, Suda K, Takaku F (1981) Effect of nitrous oxide on human bone marrow cells and its synergistic effect with methionine and methotrexate on functional folate deficiency. Cancer Res 41: 4698
18. Kano Y, Sakamoto S, Sakuraya K, Kubota T, Kasahara T, Hida K, Suda K, Takaku F (1983) Effects of nitrous oxide on human cell lines. Cancer Res 43: 1493
19. Kroes ACM, Lindemans J, Abels J (1984) Synergistic growth inhibiting effect of nitrous oxide and cycloleucine in experimental rat leukaemia. Br J Cancer 50: 793

20. Kroes ACM, Lindemans J, Hagenbeek A, Abels J (1984) Nitrous oxide reduces growth of experimental rat leukemia. Leukemia Res 8: 441
21. Lassen HCA, Henriksen E, Neukirch F, Kristensen HS (1956) Treatment of tetanus. Severe bone marrow depression after prolonged nitrous-oxide anaesthesia. Lancet 1: 527
22. Lau KS, Gottlieb C, Wasserman LR, Herbert V (1965) Measurement of serum vitamin B12 level using radioisotope dilution and coated charcoal. Blood 26: 202
23. Lavoie A, Tripp E, Hoffbrand AV (1974) The effect of vitamin B12 deficiency on methylfolate metabolism and pteroylpolyglutamate synthesis in human cells. Clin Sci Mol Med 47: 617
24. Ludwig Breast Cancer Study Group (1983) Toxic effects of early adjuvant chemotherapy for breast cancer. Lancet 2: 542
25. Lumb M, Perry J, Deacon R, Chanarin I (1981) Changes in tissue tolates accompanying nitrous oxide-induced inactivation of vitamin B12 in the rat. Am J Clin Nutr 34: 2412
26. Mandella R, DePaola DP (1984) Measurement of tissue folates: a comparison of radioassay and microbiological methods. Nutr Res 4: 521
27. McGing P, Reed B, Weir DG, Scott JM (1978) The effect of vitamin B12 inhibition in vivo: impaired folate polyglutamate biosynthesis indicating that 5-methyltetrahydropteroylglutamate is not its usual substrate. Biochem Biophys Res Commun 82: 540
28. McKenna B, Weir DG, Scott JM (1980) The induction of functional vitamin B12-deficiency in rats by exposure to nitrous oxide. Biochim Biophys Acta 628: 314
29. McMartin KE, Virayotha V, Tephly TR (1981) High-pressure liquid chromatography separation and determination of rat liver folates. Arch Biochem Biophys 209: 127
30. Metz J, Kelly A, Sweet VC, Waxman S, Herbert V (1968) Deranged DNA synthesis by bone marrow from vitamin B12-deficient humans. Br J Haematol 14: 575
31. Moran RG, Colman PD (1984) Mammalian folyl polyglutamate synthetase: partial purification and properties of the mouse liver enyzme. Biochemistry 23: 4580
32. Moran RG, Mulkins M, Heidelberger C (1979) Role of thymidylate synthetase activity in development of methotrexate cytotoxicity. Proc Natl Acad Sci USA 76: 5924
33. Muir M, Chanarin I (1984) Conversion of endogenous cobalamins into microbiologically-inactive cobalamin analogues in rats by exposure to nitrous oxide. Br J Haematol 58: 517
34. Perry J, Chanarin I, Deacon R, Lumb M (1983) Chronic cobalamin inactivation impairs folate polyglutamate synthesis in the rat. J Clin Invest 71: 1183
35. Perry J, Chanarin I, Deacon R, Lumb M (1985) Folate polyglutamate synthetase activity in the cobalamin-inactivated rat. Biochem J 227: 73
36. Rachmilewitz D, Rachmilewitz EA, Poliack A, Hershko C (1972) Acute promyelocytic leukaemia: a report of five cases with a comment on the diagnostic significance of serum vitamin B12 determination. Br J Haematol 22: 87
37. Rupreht J, Dzoljic MR (1982) Elimination of irritating compounds during chronic exposure to gases. J Pharmacol Methods 8: 109
38. Skacel PO, Hewlett AM, Lewis JD, Lumb M, Nunn JF, Chanarin I (1983) Studies on the haemopoietic toxicity of nitrous oxide in man. Br J Haematol 53: 189
39. Solter AW, Handschumacher RE (1969) A rapid quantitative determination of deoxyribonucleoside triphosphates based on the enzymatic synthesis of DNA. Biochim Biophys Acta 174: 585
40. Van Bekkum DW, Hagenbeek A (1977) Relevance of the BN leukemia as a model for human acute myeloid leukemia. Blood Cells 3: 565
41. Washtien WL (1982) Thymidylate synthetase levels as a factor in 5-fluorodeoxyuridine and methotrexate cytotoxicity in gastrointestinal tumor cells. Mol Pharmacol 21: 723
42. Wickramasinghe SN (1981) The deoxyuridine suppression test: a review of its clinical and research applications. Clin Lab Haematol 3: 1

Received July 31, 1985/Accepted December 3, 1985

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name: Patricia Duffy | Org or A.U.: 1645 | Phone: 305-7555

Serial Number: 09/654,116 | Req. Date: ~~ASAP~~ | Date:

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Carmel etc.
Journal/Book Title:	Blood
Article Title:	
Volume (Issue):	49(6).
Pages:	~~957-970~~ 987 - 1000
Year of Publication:	1977
Publisher:	
Remarks:	

FT68042 05/30

861380

STIC Use Only Accession Number: 397778

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

rall DJ, Clegg JB: The Thalas-
mes. Oxford, Blackwell, 1972
z E, Atwater J: α-Thalassemia
an Negro. J Clin Invest 51:412-

H: Different types of alpha-
nd significance of haemoglobin
ites. Lancet 2:78-80, 1970
M, Rucknagel DL, Dublin PA
III: Trimodality in the propor-
globin G-Philadelphia in het-
idence for heterogeneity in the
nan alpha chain loci. Proc Natl
73:3633-3636, 1976
A, Lang A, White JM, Lehman
stetric history in women with
emia associated with α-thalas-
d J 4:524-526, 1972

Circulating Antibody to Transcobalamin II Causing Retention of Vitamin B_{12} in the Blood

By Ralph Carmel, Basil Tatsis, and Lynn Baril

A patient with recurrent pulmonary abscess, weight loss, and alcoholism was found to have extremely high serum vitamin B_{12} and unsaturated vitamin B_{12}-binding capacity (UBBC) levels. While transcobalamin (TC) II was also increased, most of his UBBC was due to an abnormal binding protein which carried >80% of the endogenous vitamin B_{12} and was not found in his saliva, granulocytes, or urine. This protein was shown to be a complex of TC II and a circulating immunoglobulin (IgGκ and IgGλ). Each IgG molecule appeared to bind two TC II molecules. The reacting site did not interfere with the ability of TC II to bind vitamin B_{12}, but did interfere with its ability to transfer the vitamin to cells in vitro. The site was not identical to that reacting with anti-human TC II antibody produced in rabbits. Because of this abnormal complex, ^{57}Co-vitamin B_{12} injected intravenously was cleared slowly by the patient. However, no metabolic evidence for vitamin B_{12} deficiency was demonstrable, although the patient initially had megaloblastic anemia apparently due to folate deficiency. The course of the vitamin B_{12}-binding abnormalities was followed over 4 yr and appeared to fluctuate with the status of the patient's illness. The IgG–TC II complex resembled one induced in some patients with pernicious anemia by intensive treatment with long-acting vitamin B_{12} preparations. The mechanism of induction of the antibody formation in our patient is unknown.

VITAMIN B_{12} transport involves various vitamin B_{12}-binding proteins, as recently reviewed.[1,2] Two of the serum proteins, transcobalamin (TC) I and third binder (TC III) are closely related. Their roles in vitamin B_{12} transport are not established yet, and their absence does not seem to cause metabolic difficulty.[3] TC II, the protein primarily involved in delivery of vitamin B_{12} to cells, is the more crucial serum transport protein. Its absence causes severe vitamin B_{12} abnormality.[4,5]

Patients with abnormally elevated TC levels have been frequently described. Often, it is TC I that is elevated[6-13] and, since TC I normally carries endogenous vitamin B_{12}, serum vitamin B_{12} levels are also high. High serum TC III levels have been described[14-16] and, despite the major problem of in vitro artifact,[17,18] often appear to be elevated in plasma as well.[19,20] High TC II levels[13,21-24] have been described less often and less systematically and, as with TC III elevation, have usually not been accompanied by elevated vitamin B_{12} levels. Some earlier

From the Department of Medicine, University of Southern California School of Medicine, Los Angeles, California; the Division of Hematology, Long Island Jewish Hillside Medical Center, Queens Hospital Center Affiliation, Jamaica, N.Y.; and the Department of Medicine, Wayne State University School of Medicine, and the Special Hematology Laboratory, the Grace Hospital, Detroit, Mich.

Submitted November 23, 1976; accepted February 3, 1977.

Supported in part by USPHS Grant CA 14089 from the National Cancer Institute to the LAC-USC Comprehensive Cancer Center.

Address for reprint requests: Dr. Ralph Carmel, University of Southern California School of Medicine, 2025 Zonal Avenue, Los Angeles, Calif. 90033.

reports may have overestimated TC II elevations[7,25] because TC III was not adequately separated from TC II.[16,26,27]

In this paper we present studies on a unique patient with serum vitamin B_{12} levels and unsaturated vitamin B_{12}-binding capacity (UBBC) approximately 40 times normal. The cause was found to be different from the previously mentioned TC disorders. A circulating antibody to his own TC II apparently resulted in retention of both TC II and vitamin B_{12} in the blood. In many respects the antibody resembled one induced in some patients with pernicious anemia by repeated injections of long-acting preparations of vitamin B_{12}.[28,31]

In the following presentation the antibody-TC II complex will be referred to as "abnormal binder." It is important to keep in mind that, as in most studies of vitamin B_{12}-binding proteins, the quantity and fate of binding of ^{57}Co-B_{12} is assumed to reflect the quantity and the fate of the protein in question.

CASE HISTORY

A.S., a 38-yr-old black man, was first hospitalized in April 1972 with a right apical pulmonary cavity associated with fever and early clubbing of fingernails, 30-lb weight loss on a diet of milk, crackers, and soup over a 5-mo period, and alcoholism. There was no history of vitamin B_{12} therapy, and no neurologic symptoms were elicited. Abnormal laboratory data were folate deficiency with megaloblastic anemia (hemoglobin 6.1 gm/dl), hypocholesterolemia, polyclonal hypergammaglobulinemia, and low erythrocyte glucose-6-phosphate dehydrogenase activity. At no time during this admission or in his subsequent course did his neutrophil count exceed 8800/μl. All liver chemistry tests were normal although the liver was palpable 2 cm below the right costal margin; the spleen was not palpable. A very high serum vitamin B_{12} level prompted further evaluation. The vitamin B_{12} data during this hospitalization and subsequently are all shown in Fig. 1. Evaluation of the pulmonary abscess included tomography and bronchoscopy. All cytologic and bacterial studies were unrevealing, except for the finding of "atypical cells of bronchial and histiocytic origin" on one of the many sputum samples examined cytologically.

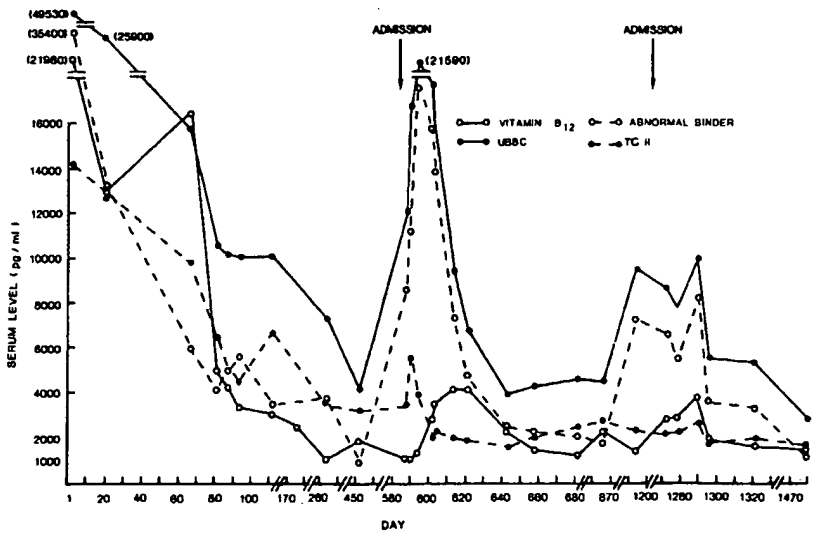
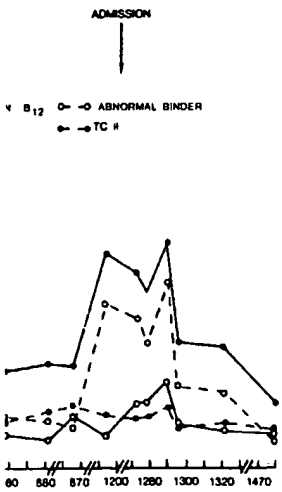


Fig. 1. Serum vitamin B_{12}, UBBC, and unsaturated binder levels of the patient. Normal serum vitamin B_{12} (150–900 pg/ml), UBBC (600–1600 pg/ml), and TC II (500–1400 pg/ml) ranges are dwarfed by the patient's levels. Abnormal binder and TC II in this graph refer to unsaturated binding only.

[25] because TC III was not

ent with serum vitamin B_{12}
(UBBC) approximately 40
from the previously men-
own TC II apparently re-
he blood. In many respects
nts with pernicious anemia
vitamin B_{12}.[28-31]
complex will be referred to
ind that, as in most studies
ite of binding of ^{57}Co-B_{12} is
otein in question.

972 with a right apical pulmonary
, 30-lb weight loss on a diet of
There was no history of vitamin
rmal laboratory data were folate
, hypocholesterolemia, polyclonal
iate dehydrogenase activity. At no
neutrophil count exceed 8800/μl.
lpable 2 cm below the right costal
amin B_{12} level prompted further
nd subsequently are all shown in
y and bronchoscopy. All cytologic
g of "atypical cells of bronchial
ined cytologically.



evels of the patient. Normal serum
C II (500–1400 pg/ml) ranges are
in this graph refer to unsaturated

A second-strength tuberculin skin test was positive and the patient was treated empirically with isoniazid, streptomycin, ethambutol, and pyridoxine after a brief course of penicillin. During the following month the patient gained weight, his hemoglobin level rose to 11.9 gm/dl and the pulmonary lesion improved rapidly. He left the hospital against advice, and his outpatient course was punctuated by very irregular antituberculous and folic acid therapy, binges of wine drinking, and intermittent mild elevations of serum GOT, GPT, lactic acid dehydrogenase, bilirubin, and alkaline phosphatase.

In December 1973 (day 582 in Fig. 1) the patient was readmitted with relapse of his earlier problems, further accompanied by diarrhea and dyspnea. He had taken no antituberculous therapy for 6 mo, but had stopped his heavy wine drinking in November 1973 following a convulsion. Although his liver was greatly enlarged, biochemical tests were normal except for minimal abnormalities of SGOT and prothrombin time. Liver biopsy showed only minimal fatty metamorphosis. There was moderate anemia of chronic disease, hypoalbuminemia, and hypocholesterolemia. The bone marrow showed 11% plasmacytosis but was normoblastic. There now a large abscess in the left lung, although all cultures and smears were again repeatedly negative. The patient was treated with penicillin, isoniazid, ethambutol, and pyridoxine. Again he showed excellent improvement. Since the course of the pneumonia was deemed not to be that of tuberculosis, he was discharged with only penicillin.

The patient was next admitted in August 1975 (day 1210) with recurrence of pneumonia in the right lung, which now showed an air-fluid level, and of diarrhea. All cultures remained negative and bronchial biopsy showed only acute and chronic inflammatory changes. The liver was not enlarged and tests of liver function were normal. Isoniazid, ethambutol, rifampin, and penicillin were given. Upper gastrointestinal x-ray studies to investigate the diarrhea were negative except for a suggestion of chronic gastritis. The patient was discharged in January 1976 without antituberculous therapy. His right pulmonary lesion has shown continued improvement, though the diagnosis remains unclear.

MATERIALS AND METHODS

All venous blood samples were allowed to clot at room temperature and were centrifuged. Plasma was also obtained once, using EDTA NaF antigoagulant[18] (Vacutainer No. 4601, Becton Dickinson & Co., Rutherford, N.J.). Saliva was collected by direct, unstimulated spitting. Random urine samples were collected without preservative. Granulocyte extracts were prepared as previously stated.[3] All specimens were stored at −20°C. Some sera were thawed and refrozen several times while preparing aliquots for experiments. Only after thawing more than 6 times was there deterioration of the vitamin B_{12} binders. The proteins were otherwise not affected by simple storage at −20°C, even after 6 mo. All experiments, unless otherwise stated, were done on minimally manipulated specimens. Depending on the experiment, cyanocobalamin labeled with cobalt-57 (^{57}Co-B_{12}) of either 15 μCi/μg or 167 μCi/μg specific activity (Amersham Searle Corp., Arlington Heights, Ill.) was used.

Serum UBBC was determined by saturating 0.1 ml of serum with excess ^{57}Co-B_{12} and, after 30 min of incubation, chromatographing in 0.1 *M* Tris-1 *M* NaCl buffer, pH 8.6, on Sephadex G-200 or G-150 gel (Pharmacia Fine Chemicals, Piscataway, N.J.) as previously described.[32] The amount of ^{57}Co-B_{12} carried in the various binder peaks was calculated, the sum being the UBBC. Results agreed closely with coated charcoal radioassay.[33] In a few experiments, different buffers were substituted in order to assess their effect on the binder; in each case the gel was also first swollen in that buffer. In some experiments, serum unlabeled with ^{57}Co-B_{12} was chromatographed and UBBC was subsequently determined on each eluate fraction by radioassay. In other studies, each such fraction was instead assayed for vitamin B_{12} content,[34] in order to determine which peaks carried the endogenous vitamin B_{12} of the serum.

Vitamin B_{12}-binding proteins were also fractionated by 2 *M* ammonium sulfate precipitation,[35] DEAE-cellulose separation,[36] and autoradiography (by exposure to x-ray film for 6 wk of cellulose acetate strips on which samples had been subjected to electrophoresis at pH 8.6).

TC fractions labeled with ^{57}Co-B_{12} were obtained by pooling gel filtration eluates, and they were concentrated by centrifuging in CF 25 Centriflo membrane cones (Amicon Corp., Lexington, Mass.). The absence of other contaminating TC in each product was confirmed by rechromatographing on the Sephadex gel. Use of such TC fractions is identified specifically below. Otherwise, whole serum was used, which, depending on the nature of the study, was sometimes first satu-

rated with nonradioactive vitamin B_{12} (the excess being removed by treating with a hemoglobin-coated charcoal pellet) in order to prevent possible undesirable transfer of $^{57}Co\text{-}B_{12}$ from components of the experiment to unsaturated binding proteins of the test serum.

Anti human saliva "R binder" antiserum was prepared in rabbits.[21] It reacted only with R binder (TC I, TC III, or granulocytic and salivary R binders) and not with TC II. Rabbit anti human TC II antiserum* was shown to react only with TC II. Precipitating anti intrinsic factor antibody was obtained from the serum of a patient with pernicious anemia and was shown to react only with intrinsic factor. Results obtained with rabbit anti-IgG, anti-IgA, anti-IgM, anti κ chain, and anti λ chain antisera (Behring Diagnostics, Woodbury, N.Y.) were confirmed with purified anti-IgG, anti-IgM, and anti-Fab antisera prepared in rabbits.†

Immune reactions were identified by change in Sephadex gel elution of the $^{57}Co\text{-}B_{12}$ bound to the binding protein in question, always comparing the pattern to a control run in which the antigen was incubated with inactive serum instead, and by autoradiography of immunoelectrophoretic or Ouchterlony immunodiffusion plates to detect radioactive arcs. In these studies, free $^{57}Co\text{-}B_{12}$ was first removed by treating the sera with hemoglobin-coated charcoal pellets. Antibody absorption studies used pure IgG† and TC fractions prepared as above. Incubation at room temperature and then at 4°C was followed by centrifugation to remove precipitated complexes before use.

In studies of pH effect, sera were acidified to pH 3 with 0.2 *N* HCl, or alkalinized to pH 10 with 0.2 *N* NaOH, and renentralized after 1 hr. To test temperature sensitivity, sera were heated to 56°C for 1 hr and then cooled. In all cases, sera were not saturated with $^{57}Co\text{-}B_{12}$ until afterward in order to avoid misinterpreting $^{57}Co\text{-}B_{12}$ release from binders as denaturation. Gel chromatography was then done to ascertain the effect of the procedures on the individual binding proteins.

The various TC fractions were incubated with neuraminidase, type VI (Sigma Chemical Co., St. Louis, Mo.) or in 5 *M* guanidine HCl to determine their effect. After dialysis at 4°C, the samples were saturated with $^{57}Co\text{-}B_{12}$ and subjected to gel chromatography and cellulose acetate electrophoresis.

In vitro mediation of $^{57}Co\text{-}B_{12}$ uptake by reticulocyte-rich red cell suspensions was tested by the method of Retief et al.[37] When $^{57}Co\text{-}B_{12}$-saturated TC fractions were used, the fraction volumes were adjusted so that each presented the same amount of bound $^{57}Co\text{-}B_{12}$ to the cells.

In vivo clearance of vitamin B_{12} was determined after injection of a subsaturating dose of 0.21 μg of sterile $^{57}Co\text{-}B_{12}$ into a forearm vein. Informed consent was obtained from the patient for the study. Venous blood was drawn from the opposite arm at timed intervals. The clearance curve may be compared to a normal one obtained in a previous study by Carmel and Herbert[3] and by others.[38,39] Some of the timed serum samples were also chromatographed on Sephadex gel to determine which binders carried the $^{57}Co\text{-}B_{12}$.

RESULTS

Serum Vitamin B_{12} and UBBC Levels (Fig. 1)

The patient's initial vitamin B_{12} level was 21,980 pg/ml and UBBC was 49,530 pg/ml. The bulk of his UBBC (71%, or 35,400 pg/ml) consisted of an abnormal binder. The patient's UBBC and abnormal binder levels were the same in plasma anticoagulated with EDTA NaF as in serum. The extremely high serum levels fell with clinical improvement, but at no time became normal. The lowest vitamin B_{12} level recorded was 1222 pg/ml (day 262) and the lowest UBBC was 2889 pg/ml (day 1480). With each clinical relapse vitamin B_{12} and UBBC values rose, though peak levels were progressively lower each time. While TC II was also elevated and its rise and fall adhered to the above pattern, the most dramatic fluctuations were in levels of the abnormal binder. TC II level exceeded that of the abnormal binder only during clinical remissions.

*Provided by Dr. Robert H. Allen, Washington University School of Medicine, St. Louis, Mo.
†Provided by Dr. Donald I. Feinstein, USC School of Medicine, Los Angeles, Calif.

by treating with a hemoglobin-
transfer of $^{57}Co\text{-}B_{12}$ from com-
est serum.
abbits.[21] It reacted only with R
id not with TC II. Rabbit anti
Precipitating anti intrinsic factor
s anemia and was shown to react
IgG, anti-IgA, anti-IgM, anti κ
ury, N.Y.) were confirmed with
bits.†
lution of the $^{57}Co\text{-}B_{12}$ bound to
control run in which the antigen
phy of immunoelectrophoretic or
. In these studies, free $^{57}Co\text{-}B_{12}$
harcoal pellets. Antibody absorp-
/e. Incubation at room tempera-
cipitated complexes before use.
N HCl, or alkalinized to pH 10
mperature sensitivity, sera were
ere not saturated with $^{57}Co\text{-}B_{12}$
se from binders as denaturation.
rocedures on the individual bind-

, type VI (Sigma Chemical Co.,
effect. After dialysis at 4°C, the
matography and cellulose acetate

red cell suspensions was tested
fractions were used, the fraction
f bound $^{57}Co\text{-}B_{12}$ to the cells.
tion of a subsaturating dose of
sent was obtained from the pa-
site arm at timed intervals. The
a previous study by Carmel and
s were also chromatographed on

g/ml and UBBC was 49,530
l) consisted of an abnormal
er levels were the same in
l. The extremely high serum
became normal. The lowest
) and the lowest UBBC was
tamin B_{12} and UBBC values
ach time. While TC II was
ove pattern, the most dra-
inder. TC II level exceeded
issions.

hool of Medicine, St. Louis, Mo.
ıe, Los Angeles, Calif.

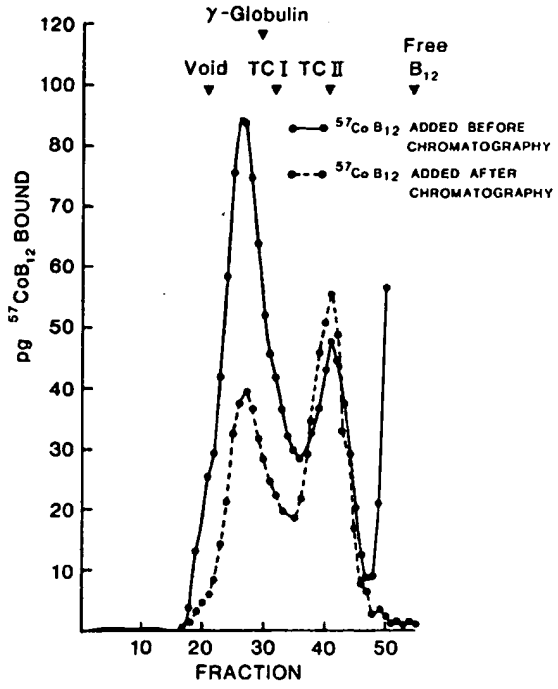


Fig. 2. Sephadex G-200 gel filtration of the patient's serum from day 588. Elution of binders without $^{57}Co\text{-}B_{12}$ is compared with the usual elution of binders carrying $^{57}Co\text{-}B_{12}$. The abnormal binder elutes between void volume and TC I.

Vitamin B_{12} was also assayed in one serum microbiologically.* The result was comparable to that of our radioassay[34] (5056 versus 4162 pg/ml), demonstrating that the high levels were not due to radioassay artifact.

Characterization of the Abnormal Vitamin B_{12}-binding Protein

Sephadex gel chromatography (Fig. 2). The binder eluted between void volume and TC I, giving a calculated Stokes radius of 7.8 nm. Comparable to the findings of others,[40] TC I had a Stokes radius of 5.2 nm and TC II of 2.8 nm. The abnormal binder was present even in serum chromatographed without $^{57}Co\text{-}B_{12}$ (Fig. 2), indicating that it did not represent aggregation induced by vitamin B_{12}. However, its binding capacity was thereby reduced 58%, suggesting the binder's partial stabilization by vitamin B_{12}. TC II was unchanged by chromatography without $^{57}Co\text{-}B_{12}$.

Cellulose acetate electrophoresis. The binder migrated as a fast γ-globulin, coming just behind TC II (β-globulin). Free $^{57}Co\text{-}B_{12}$ migrated as a slow γ-globulin.

Reaction with anti-binder antisera. Anti-TC II antiserum caused the binder to shift to the void volume on Sephadex G-200 gel chromatography (Fig. 3). The reaction was confirmed by autoradiography of Ouchterlony plate immunodiffusion, the abnormal binder giving a radioactive precipitin line of identity with TC II against anti TC II antiserum. The abnormal binder showed no reac-

**Euglena gracilis* assay performed in the laboratory of Dr. Charles A. Hall, V.A. Hospital, Albany, N.Y.

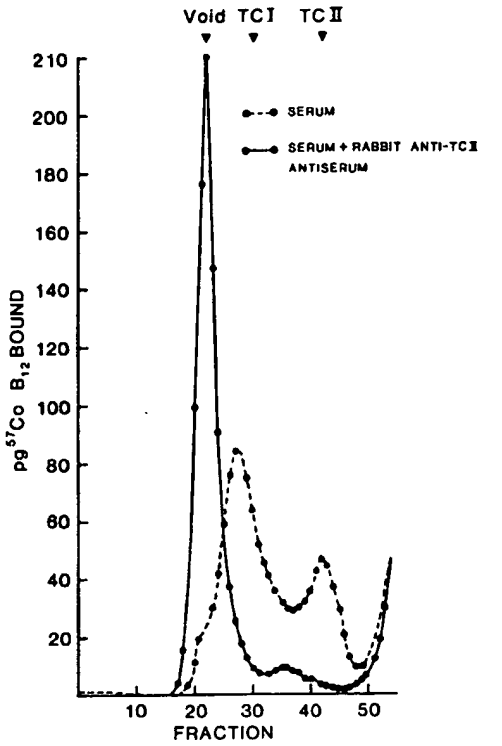


Fig. 3. Effect of rabbit anti-TC II antiserum on the patient's binders. Both TC II and abnormal binder are shifted to void volume on Sephadex G-200 gel filtration after incubation with anti-TC II.

tion by either technique with anti-salivary R binder or anti-intrinsic factor antisera.

DEAE-cellulose separation and $(NH_4)_2SO_4$ precipitation. The abnormal binder eluted from DEAE-cellulose with 0.06 *M* phosphate buffer, pH 6.3, and was precipitated in 2 *M* $(NH_4)_2SO_4$. In both these screening methods of fractionation it resembled TC II and would therefore not have been differentiated from normal TC II.

Other physicochemical and biochemical properties. The binding capacity of the abnormal binder was destroyed by heating and by acidification but was unaffected by alkalinization, generally resembling TC II in all of these features. Dialysis against 5 *M* guanidine-HCl converted most of the abnormal binder to TC II, TC II itself being unaffected by such treatment. Sephadex gel elution in low ionic strength buffer (0.01 *M* phosphate, pH 6.3) decreased both the abnormal binder and TC II greatly. The decrease was partly due to the shifting of both into the void volume fraction and partly to their retention within the gel. TC II from normal serum behaved similarly, as also found by Hom,[41] whereas TC I was unaffected. Freezing and thawing of serum with use over several months caused a progressive decrease of the abnormal binder and a slight increase of TC II, suggesting partial dissociation to TC II and partial destruction of the abnormal binder. Normal serum, similarly handled, had only a slight decrease in binding capacity. Neuraminidase did not affect the electrophoretic mobility or Sephadex gel elution of the abnormal binder or of TC II (in contrast

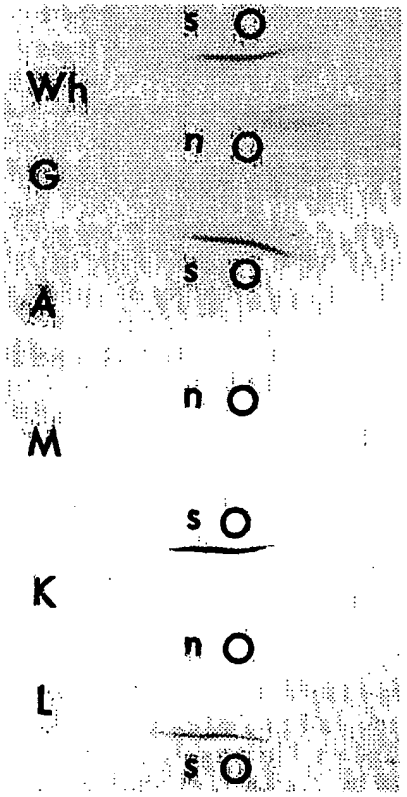


Fig. 4. Autoradiograph of immunoelectrophoretic reaction of the patient's serum with various rabbit anti-immunoglobulin antisera. The arcs represent ^{57}Co-B_{12} radioactivity, which serves as a marker for the reaction with binders. The troughs, which are very faint, contain from top to bottom: anti-whole serum antiserum (Wh), anti-IgG antiserum (G), anti-IgA antiserum (A), anti-IgM antiserum (M), anti-κ chain antiserum (K), and anti-λ chain antiserum (L). The wells (outlined circles) contain patient's serum (s) and normal control serum (n) in an alternating pattern. Normal serum reacts only with anti-whole serum, probably representing a TC II-anti-TC II reaction.

Effect of rabbit anti-TC II anti- the patient's binders. Both TC bnormal binder are shifted to me on Sephadex G-200 gel fil- ter incubation with anti-TC II.

r or anti-intrinsic factor

pitation. The abnormal phate buffer, pH 6.3, and creening methods of frac- t have been differentiated

The binding capacity of by acidification but was II in all of these features. of the abnormal binder to t. Sephadex gel elution in decreased both the abnor- tly due to the shifting of ir retention within the gel. found by Hom,[41] whereas ım with use over several al binder and a slight in- II and partial destruction dled, had only a slight de- affect the electrophoretic der or of TC II (in contrast

to its effect on TC I). Heparin and EDTA, when added to serum, had no effect on Sephadex gel elution of the abnormal binder or TC II.

Immunoglobulin characterization. The abnormal binder reacted only with rabbit anti-IgG, anti-κ chain, and anti-λ chain antisera on immunoelectrophoresis (Fig. 4), but not with anti-IgM or anti-IgA antisera. Identical results were demonstrated with Sephadex gel filtration. In addition, the binder reacted with anti-Fab antiserum in the latter system, whereas unimmunized rabbit serum had no effect on the binder. Identical results were demonstrated in serum provided to us by Dr. Henrik Olesen (Bispebjerg Hospital, Copenhagen, Denmark) from a patient with antibody to his own TC II due to therapy with depot preparations of vitamin B_{12}.

In a preliminary report of our findings,[42] we had mistakenly interpreted our patient's binder as not reacting with anti-IgG antiserum. The reasons for that initial impression were (1) presaturating anti-IgG antisera with nonradioactive vitamin B_{12} in order to saturate their own vitamin B_{12}-binding proteins and then treating with a coated charcoal pellet greatly diminished their subsequent reaction with the abnormal binder, and (2) reaction with the abnormal binder could be blocked not only by absorbing the anti-IgG antiserum with human IgG, but also by absorbing with TC II. The explanation for the first set of findings remains unclear but was due entirely to the charcoal treatment. Whatever the cause, anti-Fab reaction was not similarly blocked, suggesting that the prob-

lem was peculiar to only some antisera. The block of anti-IgG reactivity by TC II absorption turned out to be due to the unexpected contamination of the normal TC II fraction by IgG fragments. Thus, TC II prepared from an agammaglobulinemic patient did not block anti-IgG reaction with the abnormal binder. Furthermore, TC II itself did not react with the anti-IgG antiserum, indicating that the antiserum was not contaminated with anti-TC II.

Binding of endogenous vitamin B_{12}. More than 80% of the patient's endogenous vitamin B_{12} was carried in the abnormal binder peak on gel chromatography. TC I, which normally carries most vitamin B_{12}, carried only 15% 20%. The rest was bound to TC II. DEAE-cellulose fractionation confirmed these findings.

Binding avidity. The abnormal binder appeared to bind vitamin B_{12} more avidly than did TC II. When subsaturating amounts of ^{57}Co-B_{12} were added to serum in which the abnormal binder constituted 79% and TC II 21% of the UBBC, the abnormal binder bound 96% of the ^{57}Co-B_{12} and TC II only 4%.

Effect of the patient's serum on other binders. The patient's serum was presaturated with nonradioactive vitamin B_{12} to prevent simple transfer of ^{57}Co-B_{12} from test sera to his abnormal binder. Incubation of the patient's serum with various sera or with normal TC II consistently shifted the TC II to the abnormal binder peak (Fig. 5). His serum had no such effect on TC I, salivary R binder, or intrinsic factor. Nor, incidentally, did his serum react with rabbit "TC II," the predominant vitamin B_{12}-binding protein in rabbit serum which behaves like human TC II on Sephadex gel filtration. Other sera, used as controls, had no effect on TC II or TC I, nor did the patient's granulocyte extract.

In another set of experiments, the patient's serum was first filtered through Sephadex G-150 gel. Each eluate fraction was then incubated with normal TC II labeled with ^{57}Co-B_{12}. The mixtures were each rechromatographed and the amount of TC II shifted to the abnormal binder position was quantitated.

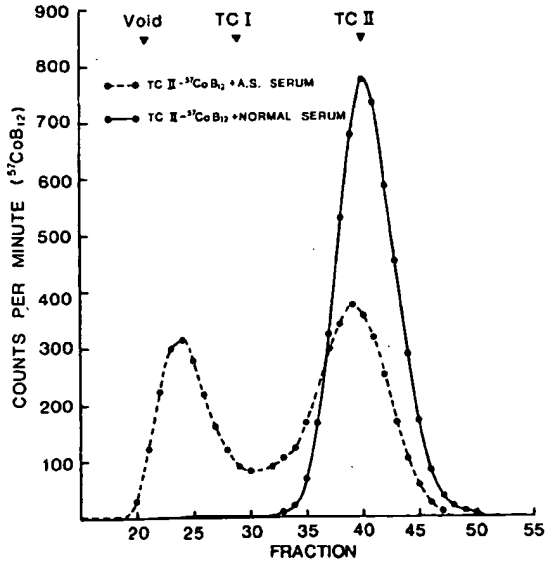


Fig. 5. Effect of the patient's serum on normal TC II (Sephadex G-150 gel chromatography). Lack of effect of normal serum on TC II is shown as a control. Both sera were first saturated with nonradioactive vitamin B_{12} in order to avoid the possibility of transfer of the ^{57}Co-B_{12} from TC II to their binding proteins.

of anti-IgG reactivity by
cted contamination of the
C II prepared from an
reaction with the abnor-
th the anti-IgG antiserum,
ith anti-TC II.
% of the patient's endog-
r peak on gel chromatog-
12, carried only 15% 20%.
tionation confirmed these

to bind vitamin B_{12} more
of ^{57}Co-B_{12} were added to
% and TC II 21% of the
B_{12} and TC II only 4%.
e patient's serum was pre-
simple transfer of ^{57}Co-B_{12}
f the patient's serum with
d the TC II to the abnor-
effect on TC I, salivary R
is serum react with rabbit
ein in rabbit serum which
1. Other sera, used as con-
ent's granulocyte extract.
was first filtered through
n incubated with normal
h rechromatographed and
position was quantitated.

Fig. 5. Effect of the patient's serum on normal TC II (Sephadex G-150 gel chromatography). Lack of effect of normal serum on TC II is shown as a control. Both sera were first saturated with nonradioactive vitamin B_{12} in order to avoid the possibility of transfer of the ^{57}Co-B_{12} from TC II to their binding proteins.

Table 1. Mediation of Vitamin B_{12} Uptake by Reticulocytes

	Percent of Added ^{57}Co-B_{12} Taken Up	
Whole serum		
Normal serum	5.4	
Chronic myelogenous leukemia serum	2.8	
Patient's serum	2.0	
	Experiment A	Experiment B*
TC fractions		
Patient's TC II†	9.0	3.6
Patient's abnormal binder	2.4	2.4
Chronic myelogenous leukemia TC I	1.7	1.2
Saline	2.7	

*In this set of experiments, the differences in endogenous content of (nonradioactive) vitamin B_{12} among the various TC fractions was neutralized by prior addition of nonradioactive vitamin B_{12} to the fractions. Thus the vitamin B_{12} contents as well as the UBBC of TC II and abnormal binder in these experiments were identical. The result was a decrease in TC II–mediated uptake, yet TC II still promoted uptake better than did the abnormal binder. (The values given represent the average of three experiments.)

†In a separate experiment normal TC II was shown to behave identically.

Peak-shifting ability was found within that elution area of the patient's serum corresponding to his abnormal binder. The shifting effect was less in serum obtained during clinical remission when vitamin B_{12} and UBBC were also lower and appeared to parallel those levels. Serum repeatedly frozen and thawed lost its ability to shift normal TC II.

Mediation of cell uptake (Table 1). The patient's serum mediated uptake of ^{57}Co-B_{12} by human reticulocyte-rich red blood cells in vitro poorly. Using binder fractions isolated by gel chromatography, his TC II was shown to behave normally, whereas his abnormal binder did not. This difference was only partly due to the difference in endogenous vitamin B_{12} content between the two binders (Table 1, experiment B). The patient's serum mediated ^{57}Co-B_{12} uptake by HeLa cells poorly too.*

Other Vitamin B_{12}-binding Proteins of the Patient

Other than its quantitative increase, the patient's TC II behaved normally electrophoretically, immunologically, on Sephadex gel chromatography, in mediating cell uptake of ^{57}Co-B_{12}, and by carrying virtually no endogenous vitamin B_{12}. TC I levels were never elevated and on gel filtration TC I was often obscured by the other two peaks. Only 15%–20% of the patient's vitamin B_{12} was carried by TC I in a serum whose vitamin B_{12} content was 3027 pg/ml.

The UBBC of two saliva specimens was 75.8 and 67.5 ng/ml (normal is 21–110 ng/ml) and was composed entirely of the normal R binder, as demonstrated immunologically, electrophoretically, and by gel filtration. The UBBC of two leukocyte extracts was 2.6 and 2.9 ng/10^8 cells (normal is 2.6–36.6 ng/10^8 cells). The binder behaved like R binder by the same criteria as above. Urine

*Performed in the laboratory of Dr. Charles A. Hall, V.A. Hospital, Albany, N.Y.

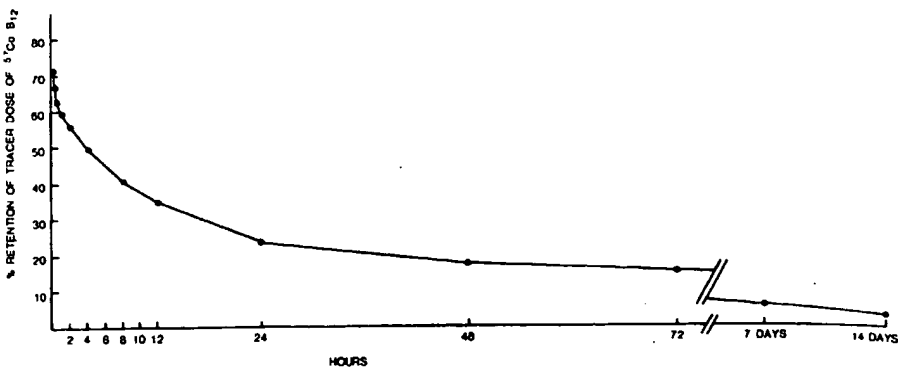


Fig. 6. Kinetics of ^{57}Co-B_{12} in vivo. The slow clearance may be compared to a normal pattern described previously.[3,38,39] The amount of ^{57}Co-B_{12} injected was taken as the 100% value. As is usually the case, no more than 71.3% was ever recovered even though blood was drawn as early as 1 min after injection. No radioactivity was found in urine at any time. This study was done near the end of the patient's second hospitalization (day 682), when the patient showed great subjective improvement and weight gain but only slight improvement in his chest x-ray. His serum vitamin B_{12} level was 1222 pg/ml and his UBBC was 4540 pg/ml, 45% of which was his abnormal binder.

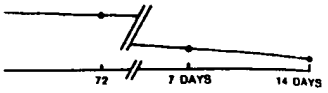
UBBC was negligible (0-46 pg/ml). Abnormal binder was not found in any of these preparations.

Clearance of ^{57}Co-B_{12} In Vivo

The patient cleared an intravenous tracer dose of ^{57}Co-B_{12} slowly (Fig. 6). The pattern was comparable to that seen in some patients with chronic myelogenous leukemia.[38,39] After 4 hr, 49.3% of the original dose was circulating; after 3 days, 15.6% was still left. Normally the values are <10% and <5%, respectively. Furthermore, this study was done at a time when his binder abnormalities were in partial remission, suggesting that clearance may have been even more abnormal during relapse. The timed serum samples were also chromatographed on Sephadex gel (Table 2). His abnormal binder carried 45% of the ^{57}Co-B_{12} initially and declined slowly; ^{57}Co-B_{12} bound to his TC II, surprisingly, also declined slowly. There was a relative increase in his TC I binding of the tracer dose after the first hour, but TC I still carried only 21% of the remaining ^{57}Co-B_{12} even after 7 days.

Table 2. Sephadex G-200 Gel Fractionation of Timed Serum Specimens Obtained During the In Vivo Clearance Study

	^{57}Co-B_{12} (cpm in sample)		
Time of Sample	Abnormal Binder	TC I	TC II
1 hr	360	51	381
4 hr	196	67	257
24 hr	131	39	114
7 days	55	28	53



may be compared to a normal
njected was taken as the 100%
recovered even though blood was
found in urine at any time. This
ation (day 682), when the patient
ly slight improvement in his chest
C was 4540 pg/ml, 45% of which

der was not found in any of

of ^{57}Co-B_{12} slowly (Fig. 6).
atients with chronic myelog-
l dose was circulating; after
re <10% and <5%, respec-
ne when his binder abnor-
learance may have been even
amples were also chromato-
l binder carried 45% of the
d to his TC II, surprisingly,
in his TC I binding of the
l only 21% of the remaining

of Timed Serum
learance Study

sample)

TC I	TC II
51	381
67	257
39	114
28	53

Miscellaneous

No evidence for vitamin B_{12} deficiency was found by testing the patient's urinary excretion of methylmalonic acid on two occasions.* The megaloblastic morphological changes, seen only initially, were apparently due to nutritional folate deficiency as suggested by low serum folate levels.

The patient's mother, the only relative available for study, had normal serum vitamin B_{12} and UBBC levels. Her serum was unable to shift normal TC II to form abnormal binder. Sera from five patients with tuberculosis (before or during antituberculous therapy), cachexia, and liver disease, and from two patients with hypergammaglobulinemia were also tested. Although most had moderate elevations of serum vitamin B_{12} and some of TC II, none had the abnormal binder seen in our patient and none could shift normal TC II.

DISCUSSION

The great elevation of UBBC and attendant retention of large amounts of vitamin B_{12} in our patient's serum were found to be due to his elaboration of a circulating antibody to his own TC II. The resultant complex-- the "abnormal binder" --was found only in his serum, and was absent from secretions such as saliva and from granulocyte extract.

The fact that the binder contained TC II was demonstrated immunologically and by its dissociating to TC II and no other TC. In addition, the binder behaved like TC II in its DEAE-cellulose elution, precipitation in 2 *M* ammonium sulfate, acid and heat denaturation, behavior on gel filtration in low ionic strength buffer, and in not being affected by neuraminidase. Many of these properties are noteworthy because they indicate that routine screening of sera by various commonly used methods would not reveal the presence of this abnormal binder.

Several authors have described apparent complexing and dissociation of TC II with various manipulations in vitro.[41,43-45] This phenomenon would not explain our patient's binder since its molecular size was quite different from those reported, and in reproducing one of these studies[43] we could not alter the binder's behavior with heparin or EDTA. Most importantly, the abnormal binder was obviously present in vivo, and its extent may even have been underestimated due to dissociation in vitro. Such dissociation (even a single pass of the complex through a Sephadex gel column produced a small TC II peak), incidentally, may explain the apparently prolonged persistence of ^{57}Co-B_{12} binding by "free" TC II in the clearance study in vivo.

The antibody component of the abnormal binder was a polyclonal IgG. Interestingly, the antibody differed from that produced in rabbits by immunization with human TC II in several respects: their sites of attachment to TC II were different, and rabbit anti-TC II formed a larger complex with TC II which eluted with the void volume fraction on Sephadex G-200 gel chromatography.

*Performed in the laboratory of Dr. Lewis A. Barness, University of South Florida College of Medicine, Tampa, Fla.

Our patient's antibody bound TC II whether or not the latter was carrying vitamin B_{12}, although whether avidity was greater for saturated or unsaturated TC II was not clear. The binding site for antibody on TC II was competitive neither with its ability to bind vitamin B_{12}, since the isolated complex could bind further vitamin B_{12}, nor with its ability to react subsequently with anti-TC II made in rabbits. However, ability to deliver vitamin B_{12} to cells in vitro appeared impaired by the antibody and clearance in vivo of vitamin B_{12} was abnormal. These findings suggest that the antibody interfered with the TC II site for attachment to cells.

If one calculates molecular size from gel filtration, the patient's binder complex corresponded to 230,000–250,000 daltons, suggesting that each IgG molecule bound two TC II molecules, the molecular weight of TC II being 38,000 by gel filtration. The apparent dissociation of TC II from antibody in 5 *M* guanidine-HCl suggests that antibody bound TC II noncovalently.

The antibody in our patient appears to be identical to that found in 29% of Danish patients with pernicious anemia treated with long-acting vitamin B_{12} preparations.[28, 31] Our patient is unique in that his antibody appeared to arise de novo since he at no time received even cyanocobalamin injections, let alone the long series of injections of the special preparations given the Danish patients. The antibody level and the vitamin B_{12} and UBBC levels rose with each exacerbation of his infectious illness, the nature of which remains unclear, and the concomitant malnutrition. The infection may have stimulated the general production of antibodies, or possibly antibody to the invading organism cross-reacted with TC II. It is interesting that in one of the Danish patients a secondary rise in vitamin B_{12} and UBBC levels coincided with a severe urinary tract infection.[31] Our patient's hepatic dysfunction did not correlate directly with the course of his abnormal binder. Nevertheless, relation of liver disease, and possibly the hypergammaglobulinemia associated with it, to this antibody must also be considered. While the complex contains immunoglobulin, its nature, of course, may not be that of true antibody formation and interaction with an antigen. Alternatively, specific autoantibody may have been stimulated by a subtle alteration of TC II or by impairment of normal vitamin B_{12} metabolism or transport.

Interestingly, despite evidence of abnormal transport, no evidence of vitamin B_{12} deficiency could be found in our patient, though subtle or selective cellular deficiency may have remained undiscovered. The closest analogy may be to patients with chronic myelogenous leukemia who, because of TC I accumulation, also have large amounts of vitamin B_{12} circulating which are poorly delivered to tissue.[37] Yet cellular depletion of vitamin B_{12}, as measured by the "deoxyuridine suppression" test,[46] has not been found in one such randomly chosen leukemic patient (R. Carmel, unpublished data). The Danish patients have also had no apparent evidence of vitamin B_{12} deficiency resulting from their antibody.[29] The presence of some TC II unbound by antibody may be sufficient for adequate vitamin B_{12} delivery to cells.

Olesen et al.[28,40] have suggested that everyone may have minute amounts of anti-TC II antibody in the serum. However, such a finding would be unexpected in the normal state. We have been unable to demonstrate even small

not the latter was carrying
for saturated or unsaturated
on TC II was competitive
the isolated complex could
act subsequently with anti-
vitamin B_{12} to cells in vitro
in vivo of vitamin B_{12} was
y interfered with the TC II

n, the patient's binder com-
gesting that each IgG mole-
ght of TC II being 38,000 by
rom antibody in 5 *M* guani-
ovalently.
ical to that found in 29% of
vith long-acting vitamin B_{12}
antibody appeared to arise
balamin injections, let alone
ations given the Danish pa-
UBBC levels rose with each
which remains unclear, and
have stimulated the general
he invading organism cross-
f the Danish patients a sec-
d with a severe urinary tract
ot correlate directly with the
on of liver disease, and pos-
h it, to this antibody must
munoglobulin, its nature, of
ion and interaction with an
have been stimulated by a
mal vitamin B_{12} metabolism

port, no evidence of vitamin
gh subtle or selective cellular
closest analogy may be to
because of TC I accumula-
ılating which are poorly de-
in B_{12}, as measured by the
ound in one such randomly
data). The Danish patients
B_{12} deficiency resulting from
bound by antibody may be

ıay have minute amounts of
h a finding would be unex-
e to demonstrate even small

amounts of antibody in several normals, treated and untreated pernicious anemia patients, and other subjects tested by gel filtration and by effect of their serum on TC II. However, careful search in our laboratory has uncovered other antibody TC complexes. Two patients were found to have antibody to their TC I, apparently arising de novo.[47] It is clear that immunoglobulin complexes with various TCs may be much more common than heretofore suspected. Since most screening methods of vitamin B_{12}-binding protein fractionation may be misleading, as we have demonstrated, identification will obviously depend on the use of appropriate techniques.

ACKNOWLEDGMENT

We wish to thank Dr. Henrik Olesen for confirming our immunologic studies of the patient's antibody, Dr. Donald Feinstein for helpful discussions, Dr. Fred Rosner for his support, the various investigators mentioned in the text and footnotes for their kind help, and Diane Owen for technical assistance.

REFERENCES

1. Allen RH: Human vitamin B_{12} transport proteins, in Brown EB (ed): Progress in Hematology, vol 9. New York, Grune & Stratton, 1975, p 57

2. Stenman UH: Vitamin B_{12}-binding proteins of R-type, cobalophilin. Characterization and comparison of cobalophilin from different sources. Scand J Haematol 14:91, 1975

3. Carmel R, Herbert V: Deficiency of vitamin B_{12}-binding alpha globulin in two brothers. Blood 33:1, 1969

4. Hakami N, Neiman, PE, Canellos GP, Lazerson J: Neonatal megaloblastic anemia due to inherited transcobalamin II deficiency in two siblings. N Engl J Med 285:1163, 1971

5. Hitzig WH, Dohmann U, Pluss HJ, Vischer D: Hereditary transcobalamin II deficiency: Clinical findings in a new family. J Pediatr 85:622, 1974

6. Miller A: The in vitro binding of cobalt^{60} labeled vitamin B_{12} by normal and leukemic sera. J Clin Invest 37:556, 1958

7. Herbert V: Diagnostic and prognostic values of measurement of serum vitamin B_{12}-binding proteins. Blood 32:305, 1968

8. Carmel R, Coltman CA Jr: Nonleukemic elevation of serum vitamin B_{12} and B_{12}-binding capacity levels resembling that in chronic myelogenous leukemia. J Lab Clin Med 78:289, 1971

9. Hall CA, Wanko M, Increased transcobalamin I in a leukemoid reaction. J Lab Clin Med 78:298, 1971

10. Waxman S, Gilbert HS: A tumor-related vitamin B_{12} binding protein in adolescent hepatoma. N Engl J Med 289:1053, 1973

11. Carmel R: Extreme elevation of serum transcobalamin I in patients with metastatic cancer. N Engl J Med 292:282, 1975

12. Nexø E, Olesen H, Norredam K, Schwartz M: A rare case of megaloblastic anaemia caused by disturbances in the plasma cobalamin binding proteins in a patient with hepatocellular carcinoma. Scand J Haematol 14:320, 1975

13. Carmel R, Eisenberg L: Serum vitamin B_{12} and transcobalamin I abnormalities in cancer. Cancer (in press)

14. Hall CA, Finkler AE: Vitamin B_{12}-binding protein in polycythemia vera plasma. J Lab Clin Med 73:60, 1969

15. Gilbert HS, Krauss S, Pasternack B, Herbert V, Wasserman LR: Serum vitamin B_{12} content and unsaturated vitamin B_{12}-binding capacity in myeloproliferative disease. Ann Intern Med 71:719, 1969

16. Carmel R: Vitamin B_{12}-binding protein abnormality in subjects without myeloproliferative disease. I. Elevated serum vitamin B_{12}-binding capacity levels in patients with leucocytosis. Br J Haematol 22:43, 1972

17. Gullberg R: Vitamin B_{12}-binding proteins in normal human blood plasma and serum. Scand J Haematol 9:639, 1972

18. Scott JM, Bloomfield FJ, Stebbins R, Herbert V: Studies on derivation of transcobalamin III from granulocytes. Enhancement by lithium and elimination by fluoride of in vitro increments in vitamin B_{12}-binding capacity. J Clin Invest 53:228, 1974

19. Stenman UH: Characterization of R-type vitamin B_{12}-binding proteins by isoelectric

focusing. III. Cobalophilin (R protein) in myeloproliferative states and leukocytosis. Scand J Clin Lab Invest 35:157, 1975

20. Carmel R: Vitamin B_{12}-binding proteins in serum and plasma in various disorders. Effect of anticoagulants. Am J Clin Pathol (in press)

21. Carmel R, Coltman CA Jr: Monocytic vitamin B_{12}-binding protein. Blood 37:360, 1971

22. Gilbert HS, Weinreb N: Increased circulating levels of transcobalamin II in Gaucher's disease. N Engl J Med 295:1096, 1976

23. Kane SP, Hoffbrand AV, Allen RH, Neale G: A familial abnormality of circulating vitamin B_{12} binding proteins: Occurrence in a family of high serum concentrations of transcobalamin II. Br J Haematol 33:249, 1976

24. Rachmilewitz B, Rachmilewitz M: Serum transcobalamin II levels in acute leukemia and lymphoma. Isr J Med Sci 12:583, 1976

25. Grassmann R, Retief FP: Serum vitamin B_{12}-binding proteins in kwashiorkor. Br J Haematol 17:237, 1969

26. Lawrence C: The heterogeneity of the high molecular weight B_{12} binder in serum. Blood 33:899, 1969

27. Bloomfield FJ, Scott JM: Identification of a new vitamin B_{12} binder (transcobalamin III) in normal human serum. Br J Haematol 22:33, 1972

28. Olesen H, Hom BL, Schwartz M: Antibody to transcobalamin II in patients treated with long acting vitamin B_{12} preparations. Scand J Haematol 5:5, 1968

29. Schwartz M, Bastrup-Madsen P: A new vitamin B_{12}-binding protein in serum causing excessively high serum vitamin B_{12} values. Scand J Haematol 5:35, 1968

30. Hom BL, Olesen H, Schwartz M: Turnover of ^{57}Co-labelled vitamin B_{12} Transcobalamin II and autologous ^{131}I-labelled IgG in a patient with antibody to transcobalamin II. Scand J Haematol 5:107, 1968

31. Skouby AP, Hippe E, Olesen H: Antibody to transcobalamin II and B_{12} binding capacity in patients treated with hydroxycobalamin. Blood 38:769, 1971

32. Carmel R: The vitamin B_{12}-binding proteins of saliva and tears and their relationship to other vitamin B_{12} binders. Biochim Biophys Acta 263:747, 1972

33. Gottlieb C, Lau KS, Wasserman LR, Herbert V: Rapid charcoal assay for intrinsic factor (IF), gastric juice unsaturated B_{12} binding capacity, antibody to IF and serum unsaturated B_{12} binding capacity. Blood 25:875, 1965

34. Carmel R, Coltman CA Jr: Radioassay for serum vitamin B_{12} with the use of saliva as the vitamin B_{12} binder. J Lab Clin Med 74:967, 1969

35. Carmel R: A rapid ammonium sulfate precipitation technic for separating serum vitamin B_{12}-binding proteins. Method and applications. Am J Clin Pathol 62:367, 1974

36. Silverstein E, Herbert V: Rapid determination of vitamin B_{12}-binding α- and β-globulin in serum. Blood 31:518, 1968

37. Retief FP, Gottlieb CW, Herbert V: Delivery of Co^{57} B_{12} to erythrocytes from α and β globulin of normal, B_{12}-deficient and chronic myeloid leukemia serum. Blood 29:837, 1967

38. Mollin DL, Pitney WR, Baker SJ, Bradley JE: The plasma clearance and urinary excretion of parenterally administered ^{58}Co B_{12}. Blood 11:31, 1956

39. Brody EA, Estren S, Wasserman LR: The kinetics of intravenously injected radioactive vitamin B_{12}: Studies on normal subjects and patients with chronic myelocytic leukemia and pernicious anemia. Blood 15:646, 1960

40. Olesen H, Rehfeld J, Hom BL, Hippe E: Stokes radius of ^{57}Co-labelled vitamin B_{12} Transcobalamin I and II and ^{125}I-labelled insulin estimated by Sephadex G-200 gel filtration in human plasma at 37°. Biochim Biophys Acta 194:67, 1969

41. Hom BL: Demonstration of transcobalamin II complex formation and binding to Sephadex G-200 at low ionic strength. Clin Chim Acta 18:315, 1967

42. Carmel R, Tatsis B: An unusual serum vitamin B_{12}-binding protein. Program, 17th Annual Meeting of the American Society of Hematology, Atlanta, Ga, 1974, p 96 (Abstr)

43. Cooper BA: Complexing of transcobalamin 2 and apparent combination with heparin. Blood 35:829, 1970

44. Meyer LM, Gizis EJ, Calas C: Aggregation of transcobalamin II. Proc Soc Exp Biol Med 140:1099, 1972

45. Cooper BA, Dirks J: Evidence for complexing of transcobalamin-2 in canine and human plasma and serum. Am J Physiol 224:758, 1973

46 Metz J, Kelly A, Swett VA, Waxman S, Herbert V: Deranged DNA synthesis by bone marrow from vitamin B_{12}-deficient humans. Br J Haematol 14:575, 1968

47. Carmel R, Shurafa M: Circulating immunoglobulin-transcobalamin I complex in patients with elevated serum vitamin B_{12} levels. Fed Proc 36:1121, 1977 (Abstr)

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Carmel et al
Journal/Book Title:	Arch. Biochem. Biophys
Article Title:	
Volume (Issue):	184(1)
Pages:	135-140
Year of Publication:	1977
Publisher:	
Remarks:	

QP501.A77

706805

921289

397773

STIC Use Only Accession Number:

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

174) *J. Biol. Chem.* 249, 1848-1856.
. (1943) *in* Proteins, Amino Acids
les (Cohn, E. J., and Edsall, J. T.,
144-505, Hafner, New York.
GOHLKE, J. R., AND RAO, D. S.
hemistry 6, 3510-3518.
A., JR., YIP, A. T., FLEMING, J.,
r, G. W. E. (1970) *J. Biol. Chem.*
5477.
, AND LIVINGSTON, R. (1969) *Advan.*
29-121.
AND STEIN, W. H. (1963) *Methods*
3, 819-831.
TOMCHO, L. A., AND PLAUT, G. W.
'. *Biol. Chem.* 250, 6197-6203.
H., AND HOLIDAY, E. R. (1952)
otein Chem. 7, 319-386.
L. (1959) *Arch. Biochem. Biophys.*

JR., AND KOSHLAND, D. E., JR.
iol. Chem. 237, 2493-2505.
B., AND HOLBROOK, J. J. (1973)
J. 133, 183-187.
A., AND KASSAB, R. (1968) *Biochim.*
cta 167, 317-325.
F., WACKER, W. E. C., AND VALLEE,
5) *Biochemistry* 4, 1758-1765.
, AND PLAUT, G. W. E. (1963) *Bio-*
2, 1023-1032.
., KIRKMAN, S. K., AND STEIN, J.
Biochemistry 6, 3197-3203.
V. E., AND AOGAICHI, T. (1968) *J.*
1. 243, 5572-5583.
M., AND COLMAN, R. F. (1974)
Biophys. Acta 370, 1-25.
., MAUCK, L., AND COLMAN, R. F.
iol. Chem. 249, 7942-7949.
, BROWN, D. M., AND PLAUT, G. W.
Biochemistry 3, 552-559.

ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 184, 135-140 (1977)

The Effect of Rhodium and Copper Analogs of Cobalamin on Human Cells *in Vitro*[1]

RALPH CARMEL[2] AND VOLKER B. KOPPENHAGEN

Department of Medicine, University of Southern California School of Medicine, Los Angeles, California; Department of Medicine, Wayne State University School of Medicine, and the Special Hematology Laboratory, The Grace Hospital, Detroit, Michigan; and Institut für Molekularbiologische Forschung, Stöckheim, West Germany

Received May 16, 1977

Rhodium and copper analogs of various cobalamins have been found to produce an anti-vitamin B_{12} effect in bacteria, leading us to see if similar inhibitory activity could be demonstrated for human cells. The analogs competed effectively with cyanocobalamin for binding by human serum transcobalamins. Methylrhodibalamin and 5'-deoxyadenosylrhodibalamin also competed with cyanocobalamin for serum-mediated uptake by human blood cells and bone marrow cells, though the competition was relatively weak when compared to the effective competition for transcobalamin II binding. None of the analogs affected normoblastic bone marrow cells, using deoxyuridine suppression of [^{3}H]thymidine incorporation into DNA as the index of vitamin B_{12} sufficiency. In fact, methylrhodibalamin actively corrected the abnormality in vitamin B_{12}-deficient bone marrow. However, 5'-deoxyadenosylrhodibalamin worsened the vitamin B_{12}-deficient behavior of megaloblastic bone marrow and inhibited its correction by vitamin B_{12} and may even have adversely affected one of the five normoblastic marrows tested.

The preparation of rhodium and copper analogs of vitamin B_{12} has been previously described (1-3). In those studies, a weak antimetabolite activity toward vitamin B_{12} was demonstrated by the ability of the analogs to suppress growth of *Lactobacillus leichmanii* (ATCC 7830) and *Escherichia coli* 113-3.

The present study was aimed at determining whether such analogs had a similar effect on human cells. Since the two metabolic steps known to require vitamin B_{12} in man, 5-methyltetrahydrofolate: homocysteine methyltransferase and methylmalonyl-CoA mutase, involve methylcobalamin and 5'-deoxyadenosylcobalamin, respectively (see Ref. 4 for a review), the rhodium analogs of those two cobalamins were of particular interest. Of the rhodium analogs, the methyl and 5'-deoxyadenosyl forms had also shown the greatest effect in bacteria (3). An *in vitro* marrow culture system (5, 6) was used in which vitamin B_{12} deficiency is identified by the resulting inability of deoxyuridine to normally suppress the subsequent incorporation of [^{3}H]thymidine into DNA. As established elsewhere (5, 6), the defect reflects the dependence of deoxyuridine monophosphate conversion to thymidine monophosphate on 5,10-methylenetetrahydrofolate, which becomes unavailable in folate deficiency or, secondarily, in vitamin B_{12} deficiency.

The binding of the analogs by human serum vitamin B_{12}-binding proteins and their uptake by human marrow cells and reticulocyte-rich red blood cell suspensions were also studied.

MATERIALS AND METHODS

Methylrhodibalamin, 5'-deoxyadenosylrhodibalamin, chlororhodibalamin, hydroxyrhodibalamin, and cupribalamin were prepared as previously de-

[1] A part of this study appeared in preliminary form in the Program of the 19th Annual Meeting, American Society of Hematology, Boston, Massachusetts, 1976, p. 211.

[2] Address all correspondence to Dr. Carmel at the University of Southern California School of Medicine, 2025 Zonal Avenue, Los Angeles, California 90033.

ISSN 0003-9861

scribed (1-3) and lyophilized. On the day of the experiment, the appropriate analogs were dissolved in 0.9% NaCl. In only a few cases were experiments done on previously dissolved analogs; these had been stored in the dark at −20°C for less than 1 week. Despite the relative stability of methylrhodibalamin and 5'-deoxyadenosylrhodibalamin in light compared to their corresponding cobalamins (3), all analogs were kept in foil-covered tubes and all experiments were carried out in an unlighted room. Cyanocobalamin, methylcobalamin, and 5'-deoxyadenosylcobalamin were obtained from Sigma Chemical Co., St. Louis, Missouri. Solutions of the latter two cobalamins were prepared in the dark on the day each was to be used in the marrow experiments. [^{57}Co]cyanocobalamin (^{57}Co-CNB$_{12}$),[3] of 15 μCi/μg specific activity, and [^{3}H]thymidine, of 19 mCi/μg specific activity, were obtained from Amersham/Searle Corp., Arlington Heights, Illinois.

Serum binding. One-half milliliter of normal serum (vitamin B$_{12}$-binding capacity = 1.2 ng/ml) was incubated with varying concentrations of analogs and 2 ng of ^{57}Co-CNB$_{12}$ for 30 min. Afterward, unbound ^{57}Co-CNB$_{12}$ was removed by the addition of hemoglobin-coated charcoal (7). Percentage inhibition of ^{57}Co-CNB$_{12}$ binding due to the analogs was determined by comparison with the amount of ^{57}Co-CNB$_{12}$ bound when no analogs had been added to the serum. In one set of experiments, the analogs were incubated with serum for 15 min before the addition of ^{57}Co-CNB$_{12}$. In several cases, inhibition of ^{57}Co-CNB$_{12}$ binding to the specific vitamin B$_{12}$-binding proteins was also determined by chromatographing the above mixtures on Sephadex G-200 gel as previously described (8) using 0.1 M Tris-1 M NaCl, pH 8.6.

Uptake by cells. Heparinized blood was obtained from subjects without megaloblastic anemia, whose reticulocyte counts exceeded 10%. The blood cells were washed and suspended in 0.9% NaCl containing 10 mM $CaCl_2$ as described by Retief *et al.* (9). Either 1 or 10 ng of either methylrhodibalamin or 5'-deoxyadenosylrhodibalamin was incubated for 30 min at 22°C with 1 ml of autologous serum or with 1 ml of phosphate-buffered sodium chloride, pH 7.4, as indicated. Then 1 ng of ^{57}Co-CNB$_{12}$ was added and incubated for 30 min at 37°C, followed by the addition of the red blood cell suspension and a further 30-min incubation at 37°C. After washing of the red blood cells with cold 0.9% NaCl, the radioactivity retained by the cells was determined. In one experiment, aspirated normoblastic bone marrow cells were substituted for peripheral blood cells. In all cases, results were compared to ^{57}Co-CNB$_{12}$ uptake by the cells in identical flasks from which only the analogs had been omitted.

In vitro deoxyuridine suppression test using bone marrow culture. The culture technique was a slight modification of the method of Metz *et al.* (6). Bone marrow aspirates from various patients were obtained during clinically indicated procedures. Several of the studies were done on patients who were vitamin B$_{12}$-deficient (untreated pernicious anemia with low serum vitamin B$_{12}$ levels, normal serum folate levels, and megaloblastic blood morphology). The rest of the marrow studies were all done on normoblastic marrow aspirates obtained from patients with normal serum vitamin B$_{12}$ and folate levels. The marrow was aspirated into Hank's salt solution which had been buffered with Tris to pH 7.3 and heparinized. All subsequent handling prior to incubation, including washing the cells, breaking up the clumps by straining through a 25-gauge needle, and mixing in 2:1 buffered Hank's solution:autologous serum, was done at 4°C.

Each experiment involved an untreated set of tubes as well as sets to which various combinations of analogs, 0.2 μg of cyanocobalamin, 50 μg of pteroylglutamic acid, 50 μg of 5-methyltetrahydrofolic acid, and 10^{-5} M methotrexate were added. Each set consisted of two pairs of triplicate tubes. After cell counts were done, equal volumes of the washed marrow suspension were added to each tube (along with ferrous ammonium sulfate if there was iron deficiency) and incubation was carried out for 15 min at 22°C. To one of the two pairs of triplicates in all sets of tubes was then added 0.1 ml of 0.5 μM deoxyuridine; to the second, "baseline" pair of triplicates, 0.1 ml of buffered Hank's solution was added instead. One hour of incubation at 37°C was followed by the addition of 1 μCi of [^{3}H]thymidine to every tube and incubation for 3 h (in later experiments, 2 h of incubation was shown to give identical results) at 37°C. The reaction was terminated by adding cold 0.9% NaCl and, after washing the cells, lysis, DNA extraction with cold 10% trichloroacetic acid, and liquid scintillation counting of the radioactivity of the dissolved precipitate were all carried out as described by Metz *et al.* (6). Experiments in our laboratory indicated that significant deoxyuridine suppression of subsequent [^{3}H]thymidine incorporation into DNA occurs in normoblastic cells as described by others (5, 6). Only 10% or less of "baseline" [^{3}H]thymidine incorporation occurred in the presence of deoxyuridine ("residual [^{3}H]thymidine incorporation"). In contrast, a megaloblastic defect was indicated by impaired deoxyuridine suppression, i.e., more than 20% "residual [^{3}H]thymidine incorporation". We would also like to emphasize that each set involving an additive or manipulation (e.g., each vitamin, analog, or inhibitor added) requires its own [^{3}H]thymidine incorporation "baseline" determination. Because of the variability of [^{3}H]thymidine incorporation in each set-

[3] Abbreviation used: ^{57}Co-CNB$_{12}$, [^{57}Co]cyanocobalamin.

; had been omitted.

uridine suppression test using bone

The culture technique was a slight the method of Metz *et al.* (6). Bone es from various patients were ob- linically indicated procedures. Sev- es were done on patients who were cient (untreated pernicious anemia vitamin B_{12} levels, normal serum d megaloblastic blood morphology). marrow studies were all done on arrow aspirates obtained from pa- mal serum vitamin B_{12} and folate row was aspirated into Hank's salt had been buffered with Tris to pH ized. All subsequent handling prior ıcluding washing the cells, breaking by straining through a 25-gauge xing in 2:1 buffered Hank's solu- serum, was done at 4°C.

nent involved an untreated set of sets to which various combinations μg of cyanocobalamin, 50 μg of acid, 50 μg of 5-methyltetrahydro- 10^{-5} M methotrexate were added. ted of two pairs of triplicate tubes. ts were done, equal volumes of the suspension were added to each tube ous ammonium sulfate if there was and incubation was carried out for To one of the two pairs of triplicates bes was then added 0.1 ml of 0.5 μM) the second, "baseline" pair of trip- f buffered Hank's solution was added ur of incubation at 37°C was followed of 1 μCi of [^{3}H]thymidine to every ıtion for 3 h (in later experiments, 2 was shown to give identical results) eaction was terminated by adding and, after washing the cells, lysis, ı with cold 10% trichloroacetic acid, illation counting of the radioactivity l precipitate were all carried out as letz *et al.* (6). Experiments in our cated that significant deoxyuridine subsequent [^{3}H]thymidine incorpo- IA occurs in normoblastic cells as thers (5, 6). Only 10% or less of hymidine incorporation occurred in deoxyuridine ("residual [^{3}H]thymi- ion"). In contrast, a megaloblastic ated by impaired deoxyuridine sup- nore than 20% "residual [^{3}H]thymi- ion". We would also like to empha- et involving an additive or manipu- ach vitamin, analog, or inhibitor s its own [^{3}H]thymidine incorpo- " determination. Because of the var- hymidine incorporation in each set- ting, deoxyuridine suppression with additives or manipulations may be erroneous if it is calculated by extrapolation to the entire experiment from a single "baseline" determined for the untreated set of tubes only.

RESULTS

Serum binding. All four rhodibalamin analogs and cupribalamin almost completely inhibited binding of subsequently added ^{57}Co-CNB$_{12}$ by normal serum (87–97% inhibition). Sephadex gel chromatography showed that the three analogs so tested (5'-deoxyadenosylrhodibalamin, hydroxyrhodibalamin, and cupribalamin) affected all of the vitamin B_{12}-binding fractions. Figure 1 shows, for example, the result when 2 ng of hydroxyrhodibalamin had been preincubated with 1 ml of serum.

Table I quantitates the inhibition caused by varying amounts of methylrhodibalamin and 5'-deoxyadenosylrhodibalamin when added simultaneously with ^{57}Co-CNB$_{12}$. At equal amounts by weight (i.e., 2 ng), the two rhodium analogs inhibited more than 50% of ^{57}Co-CNB$_{12}$ binding.

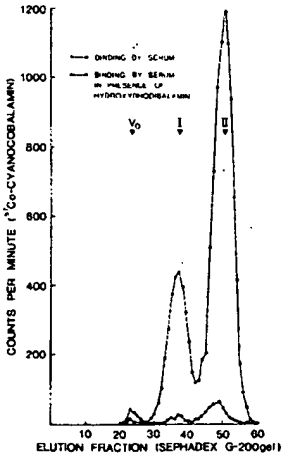


FIG. 1. Effect of hydroxyrhodibalamin on transcobalamin binding of ^{57}Co-CNB$_{12}$. The inhibition of all vitamin B_{12}-binding protein fractions by this analog is representative of all such experiments: the various analogs inhibited 90–98% of subsequent transcobalamin I and transcobalamin III binding of ^{57}Co-CNB$_{12}$ (peak I) and 86–95% of subsequent transcobalamin II binding (peak II). The normal, but still unidentified, binding protein eluting with the void volume showed 38–81% inhibition (peak V_0).

TABLE I

COMPETITION FOR SERUM BINDING BETWEEN RHODIBALAMIN ANALOGS AND ^{57}Co-CNB$_{12}$

Amount of analog added (ng)	Percentage inhibition of serum binding of 2 ng of ^{57}Co-CNB$_{12}$ by	
	Methylrhodibalamin	5'-Deoxyadenosylrhodibalamin
1	47	57
2	65	69
5	82	86
10	86	93
20	89	94
50	94	95

Uptake by blood cells. Methylrhodibalamin and 5'-deoxyadenosylrhodibalamin gave similar results (Table II). Despite the fact that both were added before ^{57}Co-CNB$_{12}$, they inhibited buffer-mediated uptake of ^{57}Co-CNB$_{12}$ weakly, whether 1 or 10 ng of analog was used. Since buffer-mediated uptake probably represents a nonspecific, mass-action phenomenon (9), this weak inhibition is not surprising. As is usual (9), ^{57}Co-CNB$_{12}$ uptake by red cells was greater in the presence of serum than in the presence of buffer. The serum-mediated uptake was also inhibited by analogs to a greater degree than was buffer-mediated uptake (Table II). However, inhibition was less than would be expected from the apparently equivalent affinity of the analogs with ^{57}Co-CNB$_{12}$ for serum binding proteins, particularly since analog was incubated with serum prior to the addition of ^{57}Co-CNB$_{12}$. Thus, while >95% of the serum-binding proteins were carrying analog instead of ^{57}Co-CNB$_{12}$, the inhibition of ^{57}Co-CNB$_{12}$ uptake did not approach this value.

In one experiment, marrow cells were substituted for peripheral blood cells. Serum-mediated uptake was again only mildly inhibited by 10 ng of analog (28% inhibition by methylrhodibalamin and 24% by 5'-deoxyadenosylrhodibalamin).

In vitro deoxyuridine suppression test using normoblastic bone marrow (Table III). In none of the experiments did any of the five analogs tested induce a vitamin B_{12}-deficient pattern. Varying concentrations of the analogs had no effect on the

residual [^{3}H]thymidine incorporation into DNA. Though 5'-deoxyadenosylrhodibalamin did appear to have some effect in one experiment, raising residual [^{3}H]thymidine incorporation from 7% to the intermediate value of 15%, the effect was not seen in other experiments at the same and higher concentrations of this analog.

In vitro deoxyuridine suppression test using vitamin B_{12}-deficient bone marrow. As expected (6), cyanocobalamin at least partially corrected the high residual [^{3}H]thymidine incorporation characteristic of megaloblastic bone marrow, as did pteroylglutamic acid. Methylrhodibalamin and 5'-deoxyadenosylrhodibalamin were the two analogs tested in this set of experiments. Methylrhodibalamin behaved just like cyanocobalamin in all bone marrow culture experiments (Fig. 2). When methylrhodibalamin was added simultaneously with cyanocobalamin or with pteroylglutamic acid, it neither inhibited nor further enhanced the corrective effect of either vitamin. The behavior of methylrhodibalamin is similar to that of methylcobalamin, which behaved just like cyanocobalamin in our system and reduced residual [^{3}H]thymidine incorporation in a vitamin B_{12}-deficient marrow from 45 to 25%, compared to a reduction to 28% by cyanocobalamin.

Unlike methylrhodibalamin, 5'-deoxyadenosylrhodibalamin did not improve the megaloblastic marrow abnormality. In fact, the abnormality was worsened by

TABLE II

INHIBITION OF UPTAKE OF ^{57}Co-CNB$_{12}$ BY RETICULOCYTES UPON PREINCUBATION WITH METHYLRHODIBALAMIN AND 5'-DEOXYADENOSYLRHODIBALAMIN

	Percentage inhibition of ^{57}Co-CNB$_{12}$ uptake by	
	Methylrhodibalamin	5'-Deoxyadenosylrhodibalamin
Buffer-mediated uptake		
1 ng of analog + buffer + 1 ng of ^{57}Co-CNB$_{12}$	8	11
10 ng of analog + buffer + 1 ng of ^{57}Co-CNB$_{12}$	19	13
Serum-mediated uptake		
1 ng of analog + serum + 1 ng of ^{57}Co-CNB$_{12}$	16	21
10 ng of analog + serum + 1 ng of ^{57}Co-CNB$_{12}$	80	80

TABLE III

EFFECT OF ANALOG ON THE DEOXYURIDINE SUPPRESSION TEST IN NORMOBLASTIC BONE MARROW CULTURES

Analog	Amount (μg)	Percentage residual [^{3}H]thymidine incorporation[a]
5'-Deoxyadenosylrhodibalamin	0.01	11 (10)
	10	8 (8); 15 (7); 11 (10)
	100	10 (10)
Methylrhodibalamin	10	9 (7); 5 (8); 6 (10); 5 (6)
	100	7 (10)
Hydroxyrhodibalamin	10	7 (7); 6 (8)
	100	12 (10)
Chlororhodibalamin	10	6 (7); 5 (8)
	100	10 (10)
Cupribalamin	10	7 (7); 8 (8)
	100	10 (10)

[a] Numbers given in parentheses are the corresponding percentage residual [^{3}H]thymidine incorporation values for "untreated" marrow cells (i.e., without added analog) for each experiment.

e cyanocobalamin in all
ılture experiments (Fig. 2).
ıodibalamin was added si-
with cyanocobalamin or
ıtamic acid, it neither in-
ther enhanced the correc-
:her vitamin. The behavior
balamin is similar to that
amin, which behaved just
lamin in our system and
ıl [^{3}H]thymidine incorpora-
min B_{12}-deficient marrow
, compared to a reduction
ocobalamin.
ıylrhodibalamin, 5′-deoxy-
alamin did not improve the
marrow abnormality. In
rmality was worsened by

ı Preincubation with
DIBALAMIN

ıntage inhibition of ^{57}Co-CNB$_{12}$ uptake by	
ylrhodi-lamin	5′-Deoxyadenosyl-rhodibalamin
8	11
19	13
16	21
80	80

LASTIC BONE MARROW CULTURES

:age residual [^{3}H]thymidine incorporation[a]
11 (10)
8 (8); 15 (7); 11 (10)
10 (10)
9 (7); 5 (8); 6 (10); 5 (6)
7 (10)
7 (7); 6 (8)
12 (10)
6 (7); 5 (8)
10 (10)
7 (7); 8 (8)
10 (10)

lual [^{3}H]thymidine incorporation experiment.

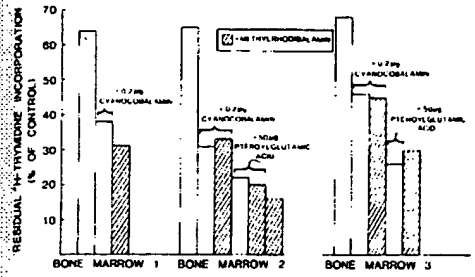


FIG. 2. Effect of methylrhodibalamin on vitamin B_{12}-deficient bone marrow in vitro. In bone marrows 1 and 2, 10 μg of methylrhodibalamin was used. In bone marrow 3, 140 μg was used. Incidentally illustrated is the better correction of the vitamin B_{12}-deficient marrow defect by pteroylglutamic acid than by vitamin B_{12}, as noted by others (6, 10). The three marrows were obtained from patients whose serum vitamin B_{12} levels ranged from 33 to 94 ng/liter (normal = 150–900), hemoglobin levels ranged from 8.1 to 11.7 g/dl, and serum folate levels ranged from 9.4 to 100 μg/liter (normal = 5–25).

this analog even when cyanocobalamin was also added (Fig. 3). This inhibition stands in contrast to the corrective effect of 5′-deoxyadenosylcobalamin on a vitamin B_{12}-deficient marrow, where residual [^{3}H]thymidine incorporation was reduced from 23 to 17%.

DISCUSSION

Our *in vitro* studies of normoblastic human bone marrow showed that none of the analogs induced a vitamin B_{12}-deficient pattern. In fact, methylrhodibalamin was able to correct the abnormality in vitamin B_{12}-deficient marrow. Thus, methylrhodibalamin behaved like methylcobalamin in this respect in man (10), a finding that contrasts with its anti-vitamin B_{12} effect in bacteria (3). However, 5′-deoxyadenosylrhodibalamin demonstrated an anti-vitamin B_{12} effect in human marrow cells. While it did not effect normoblastic marrow cells in four of five experiments, this analog clearly worsened the abnormality in vitamin B_{12}-deficient cells. Furthermore, it strikingly neutralized the corrective effect of cyanocobalamin on the vitamin B_{12}-deficient cells. Thus, 5′-deoxyadenosylrhodibalamin behaved in human cells like it did in bacteria (3) and stands in contrast to 5′-deoxyadenosylcobalamin, which has been shown here and by others (10) to have a corrective effect resembling that of cyanocobalamin in the human marrow culture system and which is hematologically effective when given to vitamin B_{12}-deficient patients (11). Although the rhodium analogs were tested separately in different marrows, the differences between the methyl and 5′-deoxyadenosyl forms of rhodibalamin were consistent and were qualitatively, not just quantitatively, different from each other. Their divergence in results stands in sharp contrast to the consistent pattern of correction by all forms of cobalamin tested.

All of the rhodium analogs tested competed well with cyanocobalamin for binding by serum vitamin B_{12}-binding proteins, there being no difference evident between transcobalamin I and transcobalamin II in this respect. Since these proteins appear to bind vitamin B_{12} along the edge of the B ring in the corrin portion with some overlap to the adjoining part of the dimethylbenzimidazole moiety (12), a metal substitution for the central cobalt would not be expected to affect binding significantly. However, despite the equiv-

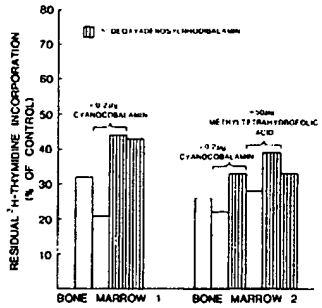


FIG. 3. Effect of 5′-deoxyadenosylrhodibalamin on vitamin B_{12}-deficient bone marrow in vitro. In bone marrow 1, 20 μg of 5′-deoxyadenosylrhodibalamin was used; in bone marrow 2, 100 μg was used. Note that this analog worsened the marrow defect even in the presence of cyanocobalamin, which is otherwise corrective. Incidentally shown with bone marrow 2, the marrow defect is not corrected by 5-methyltetrahydrofolic acid, as expected (6) and in contrast to pteroylglutamic acid (Fig. 2). These two marrows were obtained from patients whose serum vitamin B_{12} levels were 42 and 86 ng/liter, hemoglobin levels were 10.8 and 8.2 g/dl, and serum folate levels were 100 and 23.8 μg/liter.

alent affinity for binding by transcobalamin II, which is the carrier that mediates vitamin B_{12} uptake by human cells (9), subsequent uptake by blood cells and bone marrow cells appeared to be less efficient for the rhodium analogs tested than for cyanocobalamin. Nevertheless, with the comparatively greater amounts of 5'-deoxyadenosylrhodibalamin used in the bone marrow deoxyuridine suppression test (20–100 vs 0.2 μg of cyanocobalamin), competition of the analog for uptake by the cells must have been great. However, interpretation of effective uptake competition must still be cautious since cell uptake is not necessarily equivalent to entry into the cell across the membrane. Herbert commented in his review (4) on the apparent discrepancy between uptake and entry for 5'-deoxyadenosylcobalamin. Nevertheless, while some of the anti-vitamin B_{12} activity of 5'-deoxyadenosylrhodibalamin may yet conceivably result from transport interference, metabolic competition with vitamin B_{12} beyond that step seems implicated. The substitution for the central cobalt with rhodium appears to dramatically alter the behavior of this coenzyme, and the analog may prove a useful tool in studying vitamin B_{12} metabolism in man.

It is interesting that a similar rhodium substitution in methylcobalamin does not similarly affect its behavior in human cells. From the corrective effect of methylrhodibalamin in vitamin B_{12}-deficient marrow cells one might speculate that this compound can substitute for methylcobalamin as a methyl group carrier in the 5-methyltetrahydrofolate:homocysteine methyltransferase of human bone marrow, whereas in the same system such a function of vitamin B_{12} is inhibited by 5'-deoxyadenosylrhodibalamin. While transport differences between these two rhodium analogs are not completely ruled out, they appear unlikely since they behaved similarly in our binding and uptake studies. Studies with enzyme extracts of bone marrow are indicated to prove this assumption.

That methylrhodibalamin had an inhibitory effect on the growth of *E. coli* (3), presumably by inhibiting its methylcobalamin-dependent methyltransferase, is an intriguing paradox whose contrast with the analog's corrective effect in a similar metabolic step in our human marrow system is unexplained. It should also be noted that 5'-deoxyadenosylrhodibalamin was the inhibitory analog in human marrow DNA synthesis and had a greater inhibitory effect than methylrhodibalamin in *E. coli*; both systems require methylcobalamin as coenzyme (4, 13). In contrast, *L. leichmanii*, which requires 5'-deoxyadenosylcobalamin for its ribonucleotide reductase (4, 13), was inhibited by methylrhodibalamin much more than by 5'-deoxyadenosylrhodibalamin (3). Our findings thus demonstrate that the central metal as well as the particular coenzyme form are crucial in the behavior of vitamin B_{12}.

ACKNOWLEDGMENTS

We wish to acknowledge the great encouragement and support provided by the late Dr. Joseph J. Pfiffner and to thank Dr. Fritz Wagner for helpful discussions. We also thank Ms. Lynn Baril for her expert technical assistance.

REFERENCES

1. KOPPENHAGEN, V. B., AND PFIFFNER, J. J. (1971) *J. Biol. Chem.* 246, 3075–3077.
2. KOPPENHAGEN, V. B., WAGNER, F., AND PFIFFNER, J. J. (1973) *J. Biol. Chem.* 248, 7999–8002.
3. KOPPENHAGEN, V. B., ELSENHANS, B., WAGNER, F., AND PFIFFNER, J. J. (1974) *J. Biol. Chem.* 249, 6532–6540.
4. HERBERT, V. (1971) *in* The Cobalamins (Arnstein, H.R.V., and Wrighton, R. J., eds.), pp. 2–16, Churchill Livingstone, Edinburgh.
5. KILLMANN, S. A. (1964) *Acta Med. Scand.* 175, 483–488.
6. METZ, J., KELLY, A., SWETT, V. C., WAXMAN, S., AND HERBERT, V. (1968) *Brit. J. Haematol.* 14, 575–592.
7. HERBERT, V., GOTTLIEB, C. W., AND LAU, K. S. (1966) *Blood* 28, 130–132.
8. CARMEL, R. (1974). *Amer. J. Clin. Pathol.* 62, 367–372.
9. RETIEF, F. P., GOTTLIEB, C. W., AND HERBERT, V. (1966). *J. Clin. Invest.* 45, 1907–1915.
10. VAN DER WEYDEN, M. B., COOPER, M., AND FIRKIN, B. G. (1973) *Blood* 41, 299–308.
11. HERBERT, V., AND SULLIVAN, L. W. (1964) *Ann. N.Y. Acad. Sci.* 112, 855–870.
12. HIPPE, E., HABER, E., AND OLESEN, H. (1971) *Biochim. Biophys. Acta* 243, 75–82.
13. SILBER, R., AND MOLDOW, C. F. (1970) *Amer. J. Med.* 48, 549–554.

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	69/654, 114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Gams etal.
Journal/Book Title:	Proc. Soc. Exper. Biol. Med.
Article Title:	
Volume (Issue):	149(2)
Pages:	384-388.
Year of Publication:	1975
Publisher:	
Remarks:	

V.115/30

7068043

115414

STIC Use Only Accession Number: 397779

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date	Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 384–388 (1975)

Metabolism of Cyanocobalamin by L-1210 Leukemic Lymphoblasts (38813)

RICHARD A. GAMS, ELAINE M. RYEL, AND LEO M. MEYER

Hematology Division, Department of Medicine, and The Comprehensive Cancer Center, UAB School of Medicine, Birmingham, Alabama 35294 and The Hematology Section, Veterans Administration Hospital, Brooklyn, New York 11209

The cellular uptake of vitamin B-12 is a calcium-dependent process mediated by two of the recognized protein binders of vitamin B-12, gastric intrinsic factor (IF) and plasma transcobalamin II (TCII). Although the mechanism of transmembrane transport remains obscure there is evidence in a number of systems that the entire protein B-12 complex may enter the cell.

Pletch and Coffey (1) studied the uptake of Vitamin B-12 by rat liver *in vivo* after intracardiac injection of radiolabelled vitamin. They noted that intracellular B-12 was associated with a protein having the same molecular weight as TCII and suggested pinocytosis of the TCII–B-12 complex. Newmark (2) studied B-12 localization in fractionated rat kidney and also proposed entrance of TCII-bound B-12 by pinocytosis. Toporek *et al.* (3) observed that hog intrinsic factor facilitated B-12 absorption by isolated perfused rat liver with subsequent transfer of the vitamin to bile. The B-12-containing bile facilitated absorption of vitamin B-12 in a second perfused liver. This observation suggested cellular uptake of a protein B-12 complex in view of the fact that bile per se does not enhance liver uptake of vitamin B-12.

We have previously investigated the uptake of Vitamin B-12 from plasma employing an *in vitro* system of isolated murine L-1210 leukemic lymphoblasts (4). The majority of the B-12 taken up by the cells was found in the cytoplasmic soluble phase bound to a protein having the physical properties of TCII and the capacity to deliver B-12 to fresh L-1210 cells. This observation was consistent with the possibility that the entire TCII B-12 complex enters the cell. We have furthered these observations in the present study by investigating the fate of vitamin B-12 after uptake by L-1210 cells.

Materials and Methods. Cell preparation. Propagation and isolation of L-1210 lymphoblasts has previously been described (4). Cells were extracted in normal saline 6 days after intraperitoneal injection of 10^5 L-1210 cells into female BDF_1 mice. The intraperitoneal exudate was centrifuged (1650*g* for 10 min) and any contaminating erythrocytes removed by brief exposure to hypotonic saline. The cells were then washed twice with Fischer's medium for leukemic cells of mice (GIBCO 147:G, Grand Island Biological Company, Grand Island, NY) made $10^{-2}M$ in $CaCl_2$ (Fischer's–$CaCl_2$). A 4% (packed cell volume) suspension in Fischer's $CaCl_2$ was kept at 4°C (5–15 min) prior to use. Stained smears of the cells demonstrated 99%–100% L-1210 lymphoblasts which excluded trypan blue for up to 6 hr at 37°C.

Plasma preparation. Human EDTA plasma (Vacutainer, BD4739) was obtained from a healthy donor. The unsaturated B-12 binding capacity (UBBC) was determined by adding an excess (3 ng/ml) of ^{57}Co B-12 (Amersham/Searle Corp., sp act 50–150 mCi/mg), followed by dialysis against normal saline or gel filtration on Sephadex G-25 (UBBC 1.5 ng/ml).

Endogenous L-1210 B-12 binding protein. Approximately 10^9 L-1210 cells in Fischer's $CaCl_2$ were disrupted with 50 double strokes in a Dounce glass homogenizer and clarified by centrifugation. The clear cellular homogenate (20 ml) was concentrated to 2 ml by ultrafiltration employing an Amicon UM-10 membrane. To this concentrated sample was then added 1 ng of ^{57}Co B-12. After 30-min incubation at 37°C, an aliquot (0.5 ml) was subjected to gel filtration on Sephadex G-25. The remaining sample was incubated in duplicate with 10^7 L-1210 cells at 37°C for 2 hr as previously described (4). Normal saline and recalcified human plasma served as controls.

Cell uptake. Approximately 10^9 L-1210

: Lymphoblasts (38813)

LEO M. MEYER

· Center, UAB School of Medicine, dministration Hospital, Brooklyn,

isolation of L-1210 lympho- iously been described (4). :ted in normal saline 6 days ıeal injection of 10^5 L-1210 BDF_1 mice. The intraperi- was centrifuged (1650*g* for ny contaminating erythro- brief exposure to hypotonic were then washed twice with n for leukemic cells of mice , Grand Island Biological d Island, NY) made $10^{-2}M$:r's–$CaCl_2$). A 4% (packed pension in Fischer's $CaCl_2$: (5–15 min) prior to use. of the cells demonstrated 1210 lymphoblasts which blue for up to 6 hr at 37°C. *aration.* Human EDTA ner, BD4739) was obtained onor. The unsaturated B-12 / (UBBC) was determined :ess (3 ng/ml) of ^{57}Co B-12 rle Corp., sp act 50–150 /ed by dialysis against nor- filtration on Sephadex G-25 ıl).

-1210 B-12 binding protein. 0^9 L-1210 cells in Fischer's pted with 50 double strokes s homogenizer and clarified ı. The clear cellular homog- as concentrated to 2 ml by ıploying an Amicon UM-10 this concentrated sample l ng of ^{57}Co B-12. After 30- ıt 37°C, an aliquot (0.5 ml) gel filtration on Sephadex ining sample was incubated h 10^7 L-1210 cells at 37°C ously described (4). Normal :ified human plasma served

Approximately 10^9 L-1210 cells in Fischer's $CaCl_2$ were incubated with 10 ml recalcified human plasma subsaturated with 10 ng ^{57}Co B-12 for 2 or 6 hr at 37°C. The cells were then separated and washed three times with $10^{-2}M$ EDTA–normal saline, suspended in distilled water, disrupted with 50 double strokes in a Dounce glass homogenizer, and clarified by centrifugation. The incubation supernatants and clear cellular homogenates were then dialyzed overnight against distilled water. Plasma subsaturated with ^{57}Co B-12 (1 ng/ml) was incubated in the absence of cells to serve as a control.

Extraction and separation of cobalamins. Cobalamins were extracted from the dialyzed incubation supernates, cellular homogenates, and controls by the hot ethanol procedure described by Andstrand and Stahlberg (5) as modified by Linnell *et al.* (6). In brief, each sample (20 ml) was mixed with absolute ethanol (80 ml) and heated to 80°C for 20 min. The mixture was then cooled in an ice bath and filtered. Alcohol was removed from the filtrate in a rotary evaporator gradually increasing the temperature from 25°C to 40°C. The aqueous residue was then washed three times with ether (20-ml aliquots) and residual ether was removed by evaporation. The aqueous residue was then added to phenol containing 15% water (80 ml) and shaken vigorously. The aqueous phase was removed and the phenol layer washed three times with water (20-ml aliquots). The phenol layer was then mixed with acetone (70 ml) and ether (210 ml) and shaken with water (10 ml) for extraction of the cobalamins. The aqueous phase was then washed three times with equal volumes of ether. Residual ether was removed by evaporation. The samples were further concentrated by evaporation to a final volume of 1 ml. The extracted cobalamins were then separated by chromatography on SP-Sephadex C-25 as described by Tortolani *et al.* (7). The ion exchanger was initially equilibrated with sodium acetate buffer (0.05 *M*, pH 5.0) followed by repeated washing on the column with distilled water. All samples were co-chromatographed with a mixture containing 100 μg each of cyanocobalamin, adenosylcobalamin, methylcobalamin (all Calbiochem), and hydroxycobalamin (Sigma) standards.

The optical density of each fraction was scanned between 365 and 335 nm, a range chosen to encompass the λ max of each standard. The radioactivity of each fraction was determined in an automatic well scintillation counter (Nuclear-Chicago Model 1185) calibrated with ^{57}Co B-12 samples of known activity. All incubation, extraction, and separation procedures were carried out in the dark or with a red safelight to prevent photolytic degradation of B-12 analogs.

Results and Discussion. We have previously demonstrated that L1210 leukemic lymphoblasts take up vitamin B-12 when it is bound to plasma TCII (4). After homogenization of the L1210 cells the majority of the vitamin B-12 was in the cytoplasmic soluble phase apparently still bound to TCII although the possibility that the cells contained an endogenous TCII-like protein was not excluded. Preliminary experiments in our laboratory (unpublished data) indicate that isolated mitochondria demonstrate enhanced uptake of vitamin B-12 bound to TCII. A portion of such TCII bound vitamin B-12 is converted to coenzyme form by isolated mitochondria. In the present study we have investigated the fate of protein-bound vitamin B-12 after incubation with intact L-1210 cells.

Endogenous L-1210 B-12 binding protein. In an attempt to demonstrate the possible presence of endogenous B-12 binders with the characteristics of TCII in L1210 cells, cellular homogenates were incubated with vitamin B-12 and subjected to gel filtration on Sephadex G-25 as well as incubation with fresh L1210 cells. These experiments demonstrate that none of the radioactive B-12 was protein bound after such incubation (Fig. 1). Furthermore, the cellular homogenate contained no substances able to deliver radioactive B-12 to fresh L1210 cells (Table I). Thus, L1210 cells do not appear to contain vitamin B-12 binding proteins in the cytoplasmic soluble phase, nor does the homogenate enhance vitamin B-12 uptake in fresh cells.

Cellular conversion of cyanocobalamin.

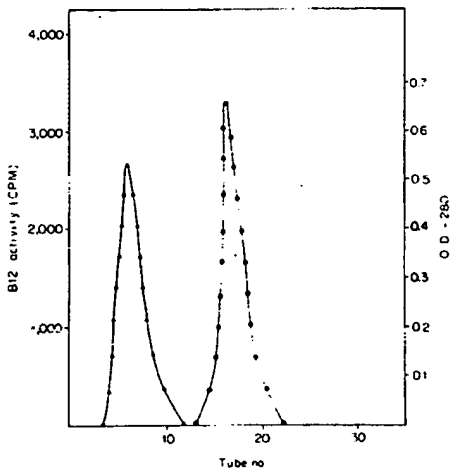


FIG. 1. Elution from Sephadex G-25 of a homogenate of L-1210 cells preincubated with 1 ng ^{57}Co-B_{12} (column size 0.9 × 25 cm, sample size 0.5 ml, fraction size 1 ml, eluant 0.5 *M* NaCl, flow rate 6 ml/hr). The clear separation of the OD 280 (▲) from the radioactivity (●) demonstrates lack of protein binding.

TABLE I. ^{57}Co B-12 UPTAKE BY L-1210 CELLS

Media[a]	Uptake (pg $B_{12}/10^7$ cells)
L-1210 homogenate	1.1
Saline	1.0
Human plasma	18.0

[a] All incubation media contain 10^{-2} *M* $CaCl_2$.

L-1210 cells were then incubated for 2 and 6 hr with ^{57}Co B-12 bound to human plasma. Dialysis of the incubation supernate and cellular homogenate resulted in negligible loss of radioactivity again suggesting that the vitamin B-12 remains protein bound. After hot ethanol extraction the various cobalamins were separated on SP-Sephadex C-25. A typical elution profile is shown in Fig. 2. The percentages of each of the cobalamins present in the 2- and 6-hr incubation supernates, cellular homogenates, and the plasma control are presented in Table II. Plasma bound B-12 incubated in the absence of cells was found to remain entirely in the cyano form. After 2-hr incubation with L1210 cells, however, approximately 6% of the B-12 was converted to other forms. After 6 hr approximately 10% was converted. Adenosylcobalamin is the major conversion product at 2 hr with methylcobalamin appearing in increased quantities at 6 hr. Peters and Hoffbrand (8) demonstrated conversion of cyanocobalamin to adenosylcobalamin in the guinea pig ileum and presented evidence that this conversion occurred in mitochondria. In their experiments approximately 20% of the vitamin was converted to a coenzyme form but a large proportion of the cyanocobalamin was absorbed unchanged. After 48 hr of incubation with L1210 cells in tissue culture, DiGirolamo *et al.* (9) found that added cyanocobalamin had been converted to adenosyl B-12 (36%), hydroxy-B-12 (28%), and methyl-B-12 (6%), while 30% remained in the cyano form. Our results after short-term incubation *in vitro* show similar trends, although no hydroxycobalamin was detected in our samples. This B-12 form may occur only after prolonged incubation, or may have resulted from hydrolysis of other forms in the tissue-culture experiment (9).

The presence of coenzyme forms of B-12 bound to protein in the incubation medium indicates counter transport of converted B-12 from the interior of the cell. Cell counts done before and after incubation remained unchanged suggesting that converted B-12 does not appear in the incubation medium because of cell lysis. Schneider *et al.* (10) studied the fate of highly purified human and rabbit TC-II *in vivo*. They found that labeled TC-II disappeared rapidly from plasma due to clearance by a variety of tissues. Labeled small-molecular-weight protein fragments soon appeared in the urine. A portion of the B-12 which had been bound to the labeled TC-II was found to reenter the circulation. These *in vivo* observations are entirely consistent with our *in vitro* studies which suggest entry of the entire TC-II cyanocobalamin complex with subsequent extracellular reappearance of coenzyme forms of the vitamin.

Conclusions. In our previous experiments (4) we demonstrated that L1210 leukemic lymphoblasts were capable of taking up vitamin B-12 when bound to transcobalamin II. Intracellular B-12 was also found to be bound to a protein with the characteristics of TCII. In the present study we have demon-

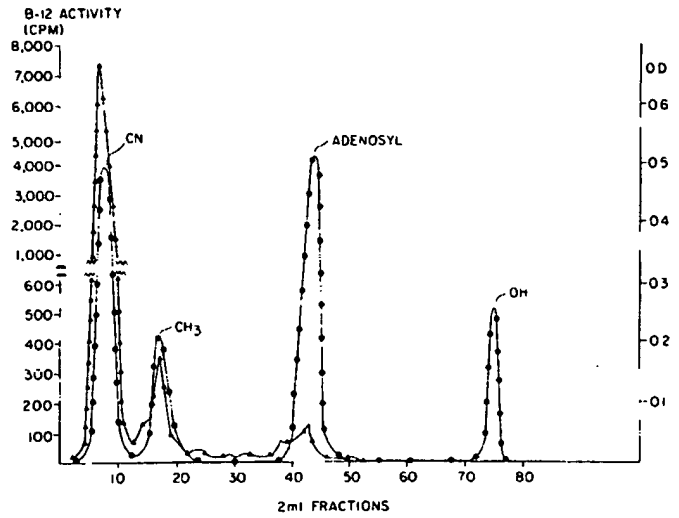


FIG. 2. Radioactivity (▲) and OD max, 365–335 nm (●) profile of the hot ethanol extract of a homogenate of L-1210 cells which had been incubated for 6 hr with TCII-bound ^{57}Co-cyanocobalamin. The extract was chromatographed with nonradioactive cobalamin standards on SP-Sephadex C-25 (column size 0.9 × 20 cm, sample size 2 ml, eluant 60 ml distilled water followed by 100 ml 0.05 *M* acetate buffer, pH 5.0. Cobalamin standards included cyanocobalamin (CN), methylcobalamin (CH_3), adenosylcobalamin (adenosyl), and hydroxycobalamin-(OH).

TABLE II. PERCENTAGE OF EACH FORM OF PROTEIN BOUND B-12 PRESENT AFTER 2 AND 6 HOURS INCUBATION WITH L-1210 CELLS.

	CN	Adenosyl	Methyl	OH
Two-hour cell supernate	94	4	2	0
Two-hour cell homogenate	94	5	1	0
Six-hour cell supernate	91	6.5	2.4	0
Six-hour cell homogenate	90	3	7	0
Six-hour plasma incubation (no cells present)	99+	0	0	0

strated that L-1210 cells do not themselves contain a TCII-like protein in the cytoplasmic soluble phase. We have further demonstrated that a portion of this protein-bound B-12 has been converted to the adenosyl and methyl forms and that such coenzyme forms may be countertransported to the exterior of the cell. These observations are consistent with our original suggestion that the entire B-12 complex crosses the plasma membrane. TCII may deliver B-12 to mitochondria where conversion to the coenzyme forms occurs. A portion of the converted vitamin appears to be transported out of the cell where it is free to recirculate.

The authors are grateful for the technical assistance of Ms. Mary Sabens, and for the generosity of Dr. Frank M. Schabel and Ms. Mary W. Trader (Southern Research Institute, Birmingham, AL) in providing BDF_1 mice harboring L1210 leukemia. This research was supported by Grant CA-13148 from the National Institutes of Health.

1. Pletsch, Q. A., and Coffey, J. W., J. Biol. Chem. **246**, 4619 (1971).
2. Newmark, P., *in* "The Cobalamins" (H. R. V. Arnstein and R. J. Wrighton, eds.), P. 79, Clowes, London (1971).
3. Toporek, M., Gizis, E. J., and Meyer, L. M., Proc. Soc. Exp. Biol. Med. **136**, 1119 (1971).

4. Ryel, E. M., Meyer, L. M., and Gams, R. A., Blood **44**, 427 (1974).
5. Lindstrand, K., and Stahlberg, K. G., Acta Med. Scand. **174**, 665 (1963).
6. Linnell, J. C., Heather, M. M., Wilson, J., and Matthews, D. M., J. Clin. Pathol. **22**, 545 (1969).
7. Tortolani, G., Biouchini, P., and Mantovani, V., J. Chromatogr. **53**, 577 (1970).
8. Peters, T. J., and Hoffbrand, A. V., Brit. J. Haematol. **19**, 369 (1970).
9. DiGirolamo, P. M., Jacobsen, D. W., and Huennekus, F. M., Amer. Soc. Hematol. (Abstr. 255), Chicago, Ill., December 1–4, 1973.
10. Schneider, R. J., Mehlman, C. S., and Allen, R. H., Amer. Soc. Hematol. (Abstr. 17), Atlanta, Ga., December 7–10, 1974.

Received Jan. 15, 1975. P.S.E.B.M., 1975, Vol. 149.

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Quadros ME.
Journal/Book Title:	Blood
Article Title:	
Volume (Issue):	86 (10).
Pages:	p 489
Year of Publication:	1995 (Nov 15)
Publisher:	
Remarks:	

RB145, A2B56
NLM

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

486

DEFECTIVE LYMPHOID DEVELOPMENT IN MICE LACKING JAK3. T. Nosaka,* J. van Deursen,* R.A. Tripp,* W.E. Thierfelder,* B.A. Witthuhn,* A.P. McMickle,* P.C. Doherty,* G. Grosveld,* J.N. Ihle. St. Jude Children's Research Hospital, Memphis, TN.

The Janus family protein tyrosine kinsases (Jaks) play a central role in signaling through cytokine receptors. While Jak1, Jak2, and Tyk2 are ubiquitously expressed, Jak3 is predominantly expressed in hematopoietic cells and uniquely associates with the common γ chain of the IL-2, 4, 7, 9, and 15 receptors. To assess the role of Jak3, we generated a *Jak3* null mutation by gene targeting in embryonic stem cells. Jak3-deficient mice showed profound thymic atrophy and severe B-cell and T-cell lymphopenia similar to human severe combined immunodeficiencies (SCID). The total numbers of thymocytes and splenocytes from $Jak3^{-/-}$ mice were reduced 10- to 100-fold and 4- to 10-fold relative to control littermates, respectively. Flow cytometric analysis of thymocytes from $Jak3^{-/-}$ mice showed decreased percentages of $CD4^+CD8^+$ cells in youngest mice at one week of age relative to controls, which increased to normal percentages with age. Thymocytes from $Jak3^{-/-}$ mice failed to respond to ConA, or PMA plus anti-CD3. Histologically, the structure of the thymuses of mutant mice was normal and contained identifiable cortical and medullary areas. Flow cytometric analysis of splenocytes from $Jak3^{-/-}$ mice revealed the dramatic reduction in $B220^+$ cells and relative increase of CD4 to CD8 single poisitive cells compared to control littermates. Splenocytes from $Jak3^{-/-}$ mice failed to respond to ConA, or PMA plus anti-CD3, and the response to LPS was greatly reduced. Also the cells showed no STAT6 DNA binding activity after IL-4 stimulation, while binding activity was readily detectable in control littermates. Bone marrow cells from $Jak3^{-/-}$ mice showed no response to LPS, IL-7, or both. The phenotype of $Jak3^{-/-}$ mice is very similar to that observed in mice which lack the common γ chain, the α chain of the IL-7 receptor, and IL-7, suggesting the importance of IL-7 receptor-mediated pathway for lymphoid development. These findings support a critical, non-redundant role of Jak3 in common γ signaling and lymphoid development. The phenotype of $Jak3^{-/-}$ mice is consistent with a classification of SCID. The results therefore support the hypothesis that the mutations and absence of Jak3 in patients with SCID are sufficient to account for their immunodeficiency. Jak3 deficient mice will serve as an important animal model for gene therapy of human SCID associated with Jak3 deficiencies.

487

SUCCESSFUL TREATMENT OF X-LINKED RECESSIVE SEVERE COMBINED IMMUNODEFICIENCY (X-SCID) BY THE IN UTERO TRANSPLANTATION OF CD34 ENRICHED PATERNAL BONE MARROW CELLS. A.W. Flake,* J.M. Puck,* G. Almeida-Porada, M.I. Evans,* M.P. Johnson,* M-G. Roncarolo, and E.D. Zanjani. Childrens Hospital of Michigan and Hutzel Hospital, Detroit, MI; NCHGR/NIH, Bethesda, MD; DNAX, Palo Alto, CA; and Department of Veterans Affairs Medical Center, University of Nevada, Reno, NV.

X-SCID is characterized by persistent infections, failure to thrive, lymphoid hypoplasia, absent mitogen response, hypogammaglobulinemia, and very low levels of T-lymphocytes. Current optimal treatment consists of early postnatal transplantation of HLA matched or T-cell depleted haploindentical bone marrow. Although usually successful (75-90%), problems of graft failure, graft versus host disease (GVHD), and delayed immunologic reconstitution, continue to compromise results. We report here the treatment of an X-SCID patient, after early prenatal diagnosis, by the in utero transplantation of CD34 enriched paternal marrow. X-inactivation analysis of maternal lymphocytes after the septic death of a previous male sibling at 7 months of age confirmed the mother to be a carrier of X-SCID. Chorionic villous sampling was performed at 11 weeks gestation of this pregnancy and the male fetus was confirmed to carry the mutated X-chromosome. After extensive non-directive counseling the parents elected to pursue prenatal transplantation. Paternal marrow harvest was performed and the cells divided into three aliquots, two of which were cryopreserved. The fresh marrow and, for later injections cryopreserved marrow, was enriched by two passages through CD34 affinity columns resulting in cell populations containing 99% $CD34^+$ cells and 0.1% $CD3^+$ cells. Three transplants were performed at 16, 17.5, and 18.5 weeks gestation of 14.8×10^6, 2×10^6, 1.8×10^6 cells respectively, by ultrasound guided intraperitoneal injection. The fetus was delivered by cesarean section at 36 weeks gestation because of fetal compromise by a double nuchal cord. Apgars were 8 and 9 and the newborn appeared normal except for a mild macular rash. Analysis of cord blood revealed absolute lymphocyte counts in the normal range with 52% $CD3^+$ cells. Molecular HLA analysis revealed a strong donor band of equal intensity to the host antigens. HLA analysis by flow cytometry confirmed the presence of mixed chimerism with 100% of the patients T-lymphocytes being of donor origin. In the first weeks of life the patients peripheral blood lymphocytes exhibited normal mitogen and allogeneic responses and the rash completely resolved with a short course of steroid therapy. At present the infant is clinically healthy with normal growth and no evidence of GVHD. In utero transplantation appears to offer advantages over postnatal transplantation in the treatment of prenatally diagnosed X-SCID. In addition, this case supports the application of in utero transplantation to selected other congenital hematopoietic diseases.

488

PHASE III RANDOMIZED STUDY OF ALL-TRANS RETINOIC ACID (ATRA) VS DAUNORUBICIN (D) AND CYTOSINE ARABINOSIDE (A) AS INDUCTION THERAPY AND ATRA VS OBSERVATION AS MAINTENANCE THERAPY FOR PATIENTS WITH PREVIOUSLY UNTREATED ACUTE PROMYELOCYTIC LEUKEMIA (APL). M.S. Tallman, J. Andersen, C.A. Schiffer, F.R. Appelbaum, J.E. Feusner, W. G. Woods, A. Ogden, H. Weinstein, L. Shepherd, J.M. Rowe, P.H. Wiernik, for the ECOG, CALGB, SWOG, CCSG, POG and NCIC-CTG. Northwestern University Medical School, Chicago, IL and ECOG, Brookline, MA.

Between April 1992 and February 1995, 401 pts with previously untreated APL were randomized to receive induction with either ATRA, 45 mg/m^2/day orally, or 1-2 cycles of conventional chemotherapy with D, 45 mg/m^2/day IV bolus days 1-3, plus A, 100 mg/m^2/day by continuous intravenous (IV) infusion days 1-7 (DA). All pts achieving complete remission (CR) were scheduled to receive two cycles of consolidation chemotherapy consisting of one cycle of DA followed by A, 2 gm/m^2 IV over one hour every 12 hours days 1-4, plus D, 45 mg/m^2/day IV bolus days 1-2. Pts on both arms were then randomized to receive either maintenance ATRA, 45 mg/m^2/day orally for one year, or observation (obs). Information is currently available for 327 pts randomized to DA or ATRA. Eight of 164 pts (5%) randomized to ATRA crossed to DA. The CR rate was 67% on each arm. There were 22/163 (13.5%) treatment-related deaths during induction with DA and 18/164 (11%) with ATRA ($p = .50$). Among pts achieving CR, with a median follow-up of 14 months, the estimated one-year disease-free survival (DFS) is 57% on the DA arm, with 43 relapses to date among the 94 pts; the estimated one-year DFS is 92% on the ATRA arm ($p < .0001$), with 11 relapses among the 98 pts. The estimated one-year overall survival (OS) from study entry for all 327 pts is 72% for DA and 82% for ATRA (not significant). 156 pts were randomized to maintenance ATRA or obs, and to date 12 of 76 pts and 34 of 80 pts have relapsed on maintenance ATRA and obs, respectively, with an estimated one-year DFS of 77% and 56% ($p < .0001$). Based on induction→maintenance arms, there have been 8 relapses among 40 pts on DA→ATRA, 4 among 36 pts on ATRA→ATRA, and 5 of 40 pts on ATRA→obs, all significantly ($p < .00001$) less than the 29 relapses among the 40 pts who never received ATRA (DA→obs). The estimated one-year DFS was 77% for DA→ATRA, 88% for ATRA→ATRA, 89% for ATRA→obs and 29% for DA→obs. These data demonstrate that ATRA does not lead to a higher CR rate or decrease in induction mortality compared to conventional chemotherapy, but exposure to ATRA either as induction or after CR is achieved is associated with an improved one-year DFS.

489 ANEMIAS I

EPITOPE SPECIFIC MONOCLONAL ANTIBODIES (mAbs) TO HUMAN TRANSCOBALAMIN II (TCII) CAN INDUCE APOPTOSIS BY INHIBITING THE CELLULAR UPTAKE OF COBALAMIN (Cbl). Edward V. Quadros,* Patricia McLoughlin,* Sheldon P. Rothenberg, A. Charles Morgan,* Marianna Shikorska-Walker,* and Roy Walker.* Division of Hematology/Oncology, SUNY-Health Science Center and V.A. Medical Center, Brooklyn, NY; Receptagen Corporation, Edmonds, WA and the Institute for Biological Sciences, National Research Council, Ottawa, Canada

Cellular uptake of Cbl is mediated by TCII, a Cbl binding protein in the plasma. The TCII-Cbl complex binds to a cell surface receptor and is internalized by endocytosis. We have generated mAbs to human TCII and have identified the following two types of mAbs with specificity for the two functional domains of the protein. Type 1: Receptor blocking. This mAb binds holo TCII and inhibits the cellular uptake of Cbl by blocking the interaction of TCII with the membrane receptor. Type 2: Cbl blocking. This mAb binds apo TCII at or near the Cbl binding domain and as a consequence, inhibits the formation of holo TCII. To determine the effect of these antibodies on TCII-receptor mediated cellular uptake of Cbl, K562 cells were cultured in normal medium (DMEM + 10% FCS) supplemented with 1ng of TCII bound [^{57}Co]Cbl per ml. Type 1 mAb was incubated overnight with the TCII-[^{57}Co]Cbl and then added to the culture. The type 2 mAb was incubated with apo TCII to form the mAb-apo TCII complex. This complex and free [^{57}Co]Cbl were then added simultaneously to the culture. Uptake of [^{57}Co]Cbl by K-562 cells measured at 24, 48 and 72 h showed that the type 1 mAb blocked (>95%) the uptake of Cbl throughout the culture period. The type 2 mAb caused a variable but lesser decrease in the uptake of Cbl. Under these experimental conditions, cell replication was not affected. K562 cells in the above experiment were cultured in FCS which contains endogenous TCII that does not crossreact with these mAbs. However, when these cells were adapted to grow in 1% human serum containing methylfolate, homocysteine and either type 1 or type 2 mAb for 10-14 days, only the cells cultured with type 1 mAb (receptor blocking) showed prolongation of doubling time followed by apoptotic cell death as indicated by DNA strand breaks and apoptotic bodies in the nucleus. Such nuclear changes may be likened to the megaloblastic bone marrow observed in Cbl deficiency. These experiments suggest that type 1 mAb may down-regulate neoplastic cell growth by inducing cellular Cbl deficiency. Moreover, since this Cbl deficiency can be rapidly induced (in contrast to nutritionally induced Cbl deficiency), these mAbs may be useful to characterize the selective biochemical disorder that underlies the cellular and neurological consequences of Cbl deficiency.

INTER LIBRARY LOAN REQUEST FORM

QP501.J7

Borrower's Name: Patricia Duffy | Org or A.U.: 1645 | Phone: 305-7555

Serial Number: 09/654,114 | Req. Date: ASA | Date:

9184106 BIOSIS NO.: 199497192476

Hepatic cobalamin deficiency induced by hydroxycobalamin (c-lactam) treatment in rats is associated with decreased mitochondrial mRNA contents and accumulation of polycistronic mitochondrial RNAs.

AUTHOR: Leeds Frederic S; Brass Eric P(a)
AUTHOR ADDRESS: (a)Case Western Reserve Univ., Dep. Med., Div. Clinical Pharmacol., 10900 Euclid Ave., Cleveland, O**USA

JOURNAL: Journal of Biological Chemistry 269 (6):p3947-3951 1994
ISSN: 0021-9258
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Treatment of rats with hydroxycobalamin(c-lactam) (HCCL), a **cobalamin antagonist**, results in both increased hepatic mitochondrial content and the development of defects in mitochondrial ubiquinol:cytochrome c oxidoreductase and cytochrome c oxidase. The present study was designed to evaluate changes in hepatic mitochondrial RNA contents in response to HCCL treatment in rats. After 2 weeks of HCCL treatment, hepatic contents of the mature mitochondrial mRNAs (expressed normalized to 28 S rRNA) encoding subunit H of cytochrome c oxidase (CO II), subunit 1 of NADH dehydrogenase (ND1), and cytochrome b were reduced to values 40-60% of those observed in RNA from control liver tissue. In addition, HCCL induced a pronounced accumulation of high molecular weight RNA species which hybridized to mitochondrial probes and represented polycistronic RNA sequences. The polycistronic RNAs were products of the heavy strand of the mitochondrial genome, and major species demonstrated hybridization patterns consistent with identifications corresponding to the 12-16 S rRNA, 12-16 S-ND1, 16 S-ND1, and CO II-ATP synthase subunit 6 regions of the mitochondrial genome. Maximal expression of the polycistronic mitochondrial RNA was observed after 2 weeks of HCCL treatment. Thus, HCCL treatment interferes with mitochondrial RNA processing and decreases the content of mature mitochondrial mRNAs. Altered expression of the mitochondrial genome may be responsible for the decreased electron transport chain activity known to result from HCCL administration.

nplete

OTHER

Attempts														2nd
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 269, No. 6, Issue of February 11, pp. 3947-3951, 1994
Printed in U.S.A.

Hepatic Cobalamin Deficiency Induced by Hydroxycobalamin[c-lactam] Treatment in Rats Is Associated with Decreased Mitochondrial mRNA Contents and Accumulation of Polycistronic Mitochondrial RNAs*

(Received for publication, July 16, 1993, and in revised form, September 15, 1993)

Frederic S. Leeds‡ and Eric P. Brass§

From the Departments of Medicine and Pharmacology, Division of Clinical Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106-4981

Treatment of rats with hydroxycobalamin[c-lactam] (HCCL), a cobalamin antagonist, results in both increased hepatic mitochondrial content and the development of defects in mitochondrial ubiquinol:cytochrome *c* oxidoreductase and cytochrome *c* oxidase. The present study was designed to evaluate changes in hepatic mitochondrial RNA contents in response to HCCL treatment in rats. After 2 weeks of HCCL treatment, hepatic contents of the mature mitochondrial mRNAs (expressed normalized to 28 S rRNA) encoding subunit II of cytochrome *c* oxidase (CO II), subunit 1 of NADH dehydrogenase (ND1), and cytochrome *b* were reduced to values 40–60% of those observed in RNA from control liver tissue. In addition, HCCL induced a pronounced accumulation of high molecular weight RNA species which hybridized to mitochondrial probes and represented polycistronic RNA sequences. The polycistronic RNAs were products of the heavy strand of the mitochondrial genome, and major species demonstrated hybridization patterns consistent with identifications corresponding to the 12–16 S rRNA, 12–16 S-ND1, 16 S-ND1, and CO II-ATP synthase subunit 6 regions of the mitochondrial genome. Maximal expression of the polycistronic mitochondrial RNA was observed after 2 weeks of HCCL treatment. Thus, HCCL treatment interferes with mitochondrial RNA processing and decreases the content of mature mitochondrial mRNAs. Altered expression of the mitochondrial genome may be responsible for the decreased electron transport chain activity known to result from HCCL administration.

Mitochondria are complex membrane-bound organelles which serve as the site of oxidative ATP synthesis in mammalian cells. Consistent with the importance of this biologic function, cellular mitochondrial content is regulated according to tissue type and metabolic demand (1, 2). Mitochondrial content appears to be governed primarily by the rates of synthesis of organellar components, especially proteins (3, 4).

Genes encoding mitochondrial proteins are contained in both the nuclear and mitochondrial genomes (3–5), and expression of mitochondrial proteins encoded by the two genomes is often coordinated (6). The mitochondrial genome is a circular double-stranded DNA (mtDNA)[1] of approximately 16,000 base pairs which encodes 13 polypeptides of the electron transport chain, and 22 tRNAs and 2 rRNAs to support mitochondrial protein synthesis (Fig. 1). Mitochondrial RNAs (mtRNA) are transcribed as polycistronic sequences derived from either strand (designated as heavy or light strands) of the mtDNA, but each strand contains only a single transcription initiation region (3–5). These mtRNA sequences are cleaved by ribonucleases which have been only partially characterized (4, 7) to yield the mature mRNA, rRNA, and tRNA sequences. Concordant changes in mitochondrial transcription and mitochondrial biogenesis have been demonstrated under some (8, 9), but not all (10), conditions.

Use of the cobalamin antagonist hydroxycobalamin[c-lactam] (HCCL) has provided a facile model of the human methylmalonic acidurias (11, 12). HCCL administration to rats is also associated with hepatic mitochondrial proliferation as assessed by enzyme activities per g of liver or per hepatocyte (13), hepatocyte oxidative capacity (13), and ultrastructural morphology (14). In addition, liver mitochondria from rats treated with HCCL for 5 weeks manifested specific defects (per mg of mitochondrial protein) in the activities of the electron transport chain enzymes ubiquinol:cytochrome *c* oxidoreductase and cytochrome *c* oxidase while other enzyme activities were normal (15). Thus, the HCCL-treated rat provides a model of altered mitochondrial expression. The present investigation tested the hypothesis that changes in hepatic mtRNA contents are associated with the HCCL-induced elevation in mitochondrial content.

EXPERIMENTAL PROCEDURES

Animals—Male Sprague-Dawley rats (200–360 g initial weight) were used in all experiments. *In vivo* treatment of rats with HCCL was accomplished as detailed previously (11–13) by implantation of subcutaneous osmotic minipumps (Alzet model 2002, Alza Corp., Palo Alto, CA) which delivered 2 μg of HCCL/h for 2 weeks. An additional rat received a replacement HCCL-loaded pump 17 days after the initial implantation and was treated with HCCL for 5 weeks. Treated rats were kept in individual wire-bottom metabolic cages for the duration of treatment as indicated in individual experiments, and control rats were

* This work was supported by National Institutes of Health Grant DK36069. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Trainee in the Medical Scientist Training Program 2 T32 GM07250 of the National Institutes of Health.

§ Burroughs Wellcome Scholar in Clinical Pharmacology. To whom all correspondence and reprint requests should be addressed: Case Western Reserve University, Dept. of Medicine, Division of Clinical Pharmacology, 10900 Euclid Ave., Cleveland, OH 44106-4981. Tel.: 216-368-4721; Fax: 216-368-3395.

[1] The abbreviations used are: mtDNA, mitochondrial DNA; mtRNA, mitochondrial RNA; HCCL, hydroxycobalamin[c-lactam]; CO II, subunit II cytochrome *c* oxidase; CYTb, cytochrome *b*; ND1, subunit 1 of NADH dehydrogenase; CO I, subunit I of cytochrome *c* oxidase; ATPase 6, subunit 6 of ATP synthase; CO II-L, light strand sequence in the CO II region of the mtDNA; ATPase 6/8, subunits 6 and 8 of the ATP synthase; CO III, subunit III of cytochrome *c* oxidase; kb, kilobase(s).

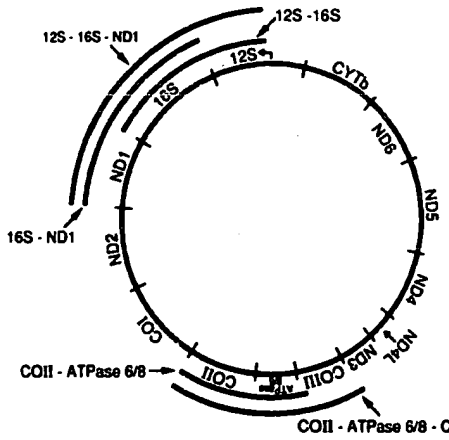


FIG. 1. **Rat mitochondrial genome.** A schematic representation of the rat mitochondrial genome is presented based on the sequence analysis of Gadaleta *et al.* (22). The order and relative sizes of the genes are indicated, with ⟵ indicating the position of the heavy-strand origin of transcription. Genes identified outside the circle are encoded by the heavy strand, whereas ND6 is encoded by the light strand. The arcs correspond to putative identification of polycistronic RNA sequences observed (see text). *ND* = NADH dehydrogenase subunits; *CO* = cytochrome *c* oxidase subunits; *ATPase* = ATP synthase subunits; and *CYTb* = cytochrome *b*.

maintained in similar cages for a period of 2 weeks. Previous experiments comparing saline pump and non-pump implanted rats as control groups demonstrated no differences in parameters of interest (data not shown). All rats were permitted *ad libitum* access to drinking water and standard rat chow (Purina Mills, Inc., St. Louis, MO).

All rats were killed at approximately the midpoint of their lights-on diurnal cycle by decapitation under ether anesthesia. Liver tissue was excised and immediately placed in liquid nitrogen. Tissues were stored at −70 °C until use.

Biochemical Assays—Frozen liver tissue (20–60 mg) was prepared using a motorized glass-on-glass homogenizer in 1 ml of water (for citrate synthase assay) or 1 ml of 0.2% Triton X-100 in 1 M ammonium hydroxide (for DNA quantitation). Citrate synthase activity was measured by the method of Srere (16) and activity expressed per g of wet weight tissue. Total liver DNA content was measured by the method of Labarca and Paigen (17) using a bisbenzimidazole concentration of 10 ng/ml.

RNA Isolation and Analysis—Total RNA was isolated from liver using the guanidinium acid-phenol extraction method of Chomczynski and Sacchi (18) except that all precipitations were performed overnight at −20 °C. Liver tissue (approximately 100 mg) was weighed and homogenized in 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% *n*-laurylsarcosine, 0.1 M β-mercaptoethanol using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) on a setting of 7 for 30 s. RNA content in the final preparation was quantified based on absorption at 260 nm, and preparations demonstrated ratios of absorbance at 260 *versus* 280 nm of greater than 1.8.

RNA was size-fractionated by electrophoresis in 1.0% agarose, 2.2 M formaldehyde horizontal gels run for 12–16 h at 15 V. RNA was transferred onto Zeta-Probe nylon membranes (Bio-Rad) by capillary blotting according to the manufacturer's directions except that 20 × SSC (0.15 M sodium chloride, 0.015 M sodium citrate) was used at the bottom of the transfer container. The membrane was stained with methylene blue to verify efficient transfer (19) and fixed by baking at 1–2 h at 80 °C. The 28 S rRNA methylene blue-stained band was quantitated by reflectance densitometry. Membranes were then destained by agitation in 300 ml of 0.2 × SSC, 1% sodium dodecyl sulfate (SDS) for 30 min.

RNA membranes were probed by hybridization with ^{32}P-labeled cDNA probes according to the directions of the membrane manufacturer (50% formamide protocol for cDNA probes, except 0.2 × SCC was used for the final wash; oligonucleotide protocol for ^{32}P-labeled oligonucleotides). Hybridizing bands were visualized by autoradiography (XAR-5 film, Eastman Kodak, Rochester, NY) or phosphorimaging (PhosphorImager, Molecular Dynamics, Inc. Sunnyvale, CA). Quantitations of phosphorimaged bands was accomplished via pixel densitometry, using the Image Quant software package (Molecular Dynamics, Inc.). Quantitation of bands of interest were normalized to the 28 S rRNA band from the same lane. Both reflectance densitometry and phosphorimaging were demonstrated to yield linear responses with respect to RNA loading in the range used for the current studies (data not shown). For re-use, membranes were stripped of probe by two consecutive 20-min incubations in 500 ml of 0.1 × SCC, 1% SDS at 95–100 °C. Sizes of RNAs of interest were estimated based on standard curves derived from RNA markers (RNA MWI, Boehringer Mannheim) by interpolation. Subsequently during routine experiments, bands of interest were monitored on any gels without markers based on mobilities of the 28 and 18 S rRNAs as well as the band of interest.

Probes—The cDNA probe for subunit II of cytochrome *c* oxidase (CO II) was isolated as the *Eco*RI-*Hin*dIII fragment from the Bluescript construct provided by C. M. Van Itallie (20). cDNA probes for 16 S rRNA and cytochrome *b* (CYTb) were prepared as the *Eco*RI fragments of Bluescript constructs originally prepared by Howard Zalkin and subcloned by C. M. Van Itallie (21). The cDNA probe for subunit 1 of NADH dehydrogenase (ND1) was the *Bam*HI fragment from plasmid pKSU1 prepared by Durwood Ray (Memorial Medical Center Cancer Institute, Tulsa, Oklahoma). CO II, 16 S RNA, and CYTb probes were cloned from rat; the ND1 probe was from mouse. cDNA probes were radiolabeled with 50 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; DuPont NEN) using a random-primer labeling kit (Oligolabeling kit, Pharmacia LKB Biotechnology Inc.). Approximately 50 ng of probe template was labeled for each hybridization experiment. Probe-specific activities were typically 10^8-10^9 dpm/μg.

Oligonucleotide probes were synthesized via the standard phosphoramidite method in the Molecular Biology Core Facility, Case Western Reserve University, Cleveland, OH. Probes were designed based on the rat mtDNA sequence published by Gadaleta *et al.* (22). The 12 S rRNA probe corresponded to mtDNA positions 540–501, the CO I probe 6096–6057, the ATPase probe 8200–8161, and the COII-L probe 7310–7349. For each hybridization, 10 pmol (approximately 120 ng) of oligonucleotide was labeled with 150 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, DuPont NEN) using a 5′-end labeling kit (Promega, Inc., Madison, WI). Under these conditions labeling was approximately quantitative with probe-specific activities typically approximating that of the [γ-^{32}P]ATP.

Synthesis of HCCL—Cobalamin[c-lactam] was synthesized by a modification of the method of Bonnet *et al.* (23). Briefly, cyanocobalamin (5 mg/ml) was dissolved in 0.1 N NaOH and incubated at 100 °C for 10 min with constant aeration. The solution was then adjusted to pH 7.0 with KH_2PO_4, evaporated under compressed air, and resuspended in water (2 mg/ml). The cyanocobalamin[c-lactam] was separated from starting material and other products using paper chromatography (Whatman 3 MM Chr) with a mobile phase of *sec*-butyl alcohol:acetic acid:water (880:8.2:425, v:v:v) containing 5 mM KCN. The cyanocobalamin[c-lactam] band was then cut and the product eluted into 50 ml of H_2O. This solution was filtered (0.2 μm) and evaporated under vacuum to yield crystals. Conversion to the hydroxy form (HCCL) was conducted as described by Dolphin (24). Formation of HCCL was confirmed by high-performance liquid chromatography and UV spectra (data not shown). The concentration of HCCL was estimated using the UV extinction coefficient for cobalamin.

Materials—Cyanocobalamin and acetyl-CoA were obtained from Sigma. Restriction enzymes were purchased from Life Technologies, Inc. All other reagents were of the highest grade available.

Data Analysis—Numeric data are presented as mean ± S.D. "*n*" refers to the number of individual animals in each group. Student's two-tailed *t* test was used to compare control and HCCL-treated groups, with $p < 0.05$ considered statistically significant. Estimation of molecular weights on Northern blots was done using a log (size) *versus* mobility (cm) linear standard curve based on the mobilities of the size standards.

RESULTS

Rats were treated with HCCL (2 μg/h by osmotic minipump) for 2 weeks. DNA content per g of liver, a sensitive marker of the nutritional status of the liver (25), was not affected by HCCL treatment (9.6 ± 0.9 *versus* 10.6 ± 0.6 μg/g in control and HCCL treated rats, respectively). In contrast, citrate synthase activity per g of liver was increased 30% by HCCL treatment as compared with control rats (6.6 ± 0.3 *versus* 8.7 ± 0.9 μmol/min/g in control and HCCL-treated rats, respectively, $p < 0.05$), verifying the HCCL-induced increase in hepatic mitochondrial content (13).

Total RNA was extracted from the livers of the control and 2-week HCCL-treated rats and Northern analysis performed for CO II mRNA, 16 S rRNA, ND1 mRNA, and CYTb mRNA.

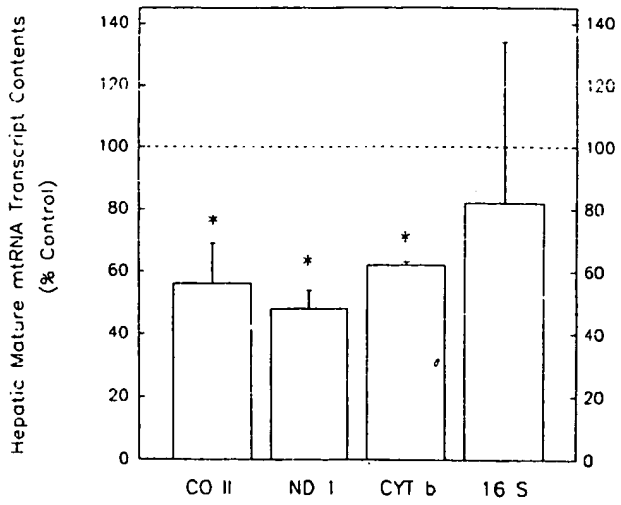


FIG. 2. **Hepatic content of mitochondrial-encoded RNAs in 2-week HCCL-treated rats.** Total RNA was isolated from liver obtained from individual control ($n = 3$) and 2-week HCCL-treated ($n = 3$) rats and Northern blot analysis conducted using a single gel for each probe. The 28 S rRNA content was quantified by reflective densitometry and used as a denominator for subsequent normalization of mature mtRNA transcripts. cDNAs for CO II, ND1, CYTb, and 16 S rRNA were used to quantify the mature RNA transcript for each gene. Values plotted are the RNA contents (mean + S.E.) for the 2-week HCCL treated rats expressed as a percentage of the mean content in the livers from control rats. *, $p < 0.05$ *versus* control (unpaired comparison with $n = 3$ in each group).

No differences were noted in the yields of total RNA isolated from control or HCCL-treated animals (data not shown). After 2-week HCCL treatment, the abundance of mature CO II mRNA in liver was reduced to 56% of control contents (Fig. 2), and the contents of ND1 and CYTb mature mRNAs were 48 and 63% (respectively) of those in liver from control rats ($p < 0.05$ for each of the three mRNAs). In contrast, the hepatic content of the 16 S rRNA was not altered by HCCL treatment.

The Northern blots utilized for the experiments summarized in Fig. 2 also demonstrated the appearance of bands corresponding to high molecular weight RNA species which hybridized to the CO II, ND1, and 16 S probes (Fig. 3). The large RNAs detected by the CO II probe were approximately 1.6, 2.5, and 4.0 kb in length, and those hybridizing to 16 S and ND1 probes were approximately 2.8 and 4.4 kb in each case (Table I, Fig. 3). A subset of these large RNAs could be visualized using RNA from control rats using long film exposures and increased RNA loading of the gels (data not shown).

Mammalian mtRNA is synthesized as a large polycistronic sequence and processed by simple intergenic cleavage with no evidence for intron excision nor trans-splicing events (3–5). Thus, if the high molecular weight RNA fragments identified in liver from HCCL-treated rats are derived from the primary polycistronic mtRNA transcript, then they must consist of sequences containing multiple contiguous open reading frames. Therefore, oligonucleotide probes corresponding to sequences flanking the CO II and 16 S genes (Fig. 1) were used to probe high-load (50 μg of total RNA/lane) Northern blots. The ATPase 6 probe hybridized to two high molecular weight bands of 1.6 and 2.5 kb (Table I). In contrast, the CO I probe hybridized only to the mature CO I mRNA. Thus both the 1.6- and 2.5-kb bands appear to contain sequence corresponding to the CO II and ATPase 6 genes. The 12 S rRNA probe detected RNAs of 2.8 and 4.4 kb, and an ND1 probe, also detected RNAs of 2.8 and 4.4 kb (Table I). An oligonucleotide probe against the light-strand RNA product in the region of the CO II gene (*i.e.* anti-sense to the coding sequence) detected no bands on the Northern blots.

To establish the time course for the HCCL-induced accretion of polycistronic mtRNAs, total RNA was isolated from the livers of control, 1-week, 2-week, and 5-week HCCL-treated rats and subjected to Northern analysis using the CO II, ND1, and 16 S probes. None of the HCCL-induced polycistronic RNAs were detectable after 1 week of treatment (Fig. 4). All of the identified polycistronic RNAs were again detected in RNA from 2-week-treated animals, with no additional accumulation observed in comparison with 5-week-treated rats.

DISCUSSION

Expression of the mitochondrial genome plays a role in the regulation of mitochondrial content in mammals (3–5). Some models of mitochondrial proliferation, including administration of thyroid hormone (21, 26), or dexamethasone (21) and exercise training (27), are associated with stimulation of mtRNA transcription. In contrast, hepatic mitochondrial proliferation in HCCL-induced methylmalonic aciduria is associated with decreased mitochondrial mature mRNA content, but marked accumulation of polycistronic mtRNAs.

In agreement with previous studies (13), the hepatic citrate synthase activity (a nuclear-encoded mitochondrial enzyme), was elevated 30% by 2 weeks of HCCL treatment. Despite the ongoing increase in mitochondrial content (documented to be progressive over 5 weeks of HCCL treatment, Ref. 13), the hepatic content (normalized to 28 S rRNA or per total RNA) of mature mRNAs for CO II, CYTb, and ND1 were reduced by 40–60%. These decreases in mitochondrial mRNAs were specific changes, as mRNAs for the nuclear-encoded proteins glyceraldehyde phosphate dehydrogenase, subunit Va of cytochrome *c* oxidase, or carnitine palmitoyltransferase II were not decreased by HCCL treatment (data not shown). In contrast with the mitochondrial mRNAs, 16 S rRNA expression was not affected by HCCL treatment. This may indicate that the expression of the mitochondrial rRNAs is under a different set of controls from that of the mRNAs (28, 29) or reflect longer kinetics of the rRNAs as compared with mRNAs (30). Thus, HCCL-treatment may not affect initiation of transcription as reflected by the stability of the 16 S rRNA content, but may instead interfere with some process specific for expression of the mature mRNAs (either synthesis or stability). The content of mitochondrial rRNAs has been proposed as a major determinant of mitochondrial translation capacity (10). Consistent with this hypothesis and the observed lack of change in hepatic 16 S rRNA content, translation capacities of hepatic mitochondria from 2-week-HCCL-treated and control rats do not differ (31).

Two-week treatment with HCCL resulted in the hepatic accumulation of high molecular weight mtRNA species which contained sequences corresponding to both open reading frames and rRNAs. Large molecular weight mtRNAs were also previously detected in hepatic RNA from HCCL-treated rats using the entire mouse mtDNA as a probe (13).[2] Polycistronic mtRNAs have been documented previously (21, 32–34) and presumably represent partially processed precursors of the mature RNAs (3–5, 33, 34). All of the HCCL-induced polycistronic RNAs evaluated are heavy-strand mtDNA products, as a light-strand probe failed to hybridize to any identifiable band, whereas heavy-strand-specific oligonucleotide probes yielded strong signals (Table I). The hybridization signals generated by the mitochondrial probes used allows inferences to be made as to the possible identity of the mtRNAs. For example, both CO II and ATPase 6 probes identified a 1.6-kb RNA. As CO II and

[2] The speculated identities for the large mtRNAs suggested by Krahenbuhl *et al.* (13) are not supported by the current data and should not be confused with the systematic evaluation presented here.

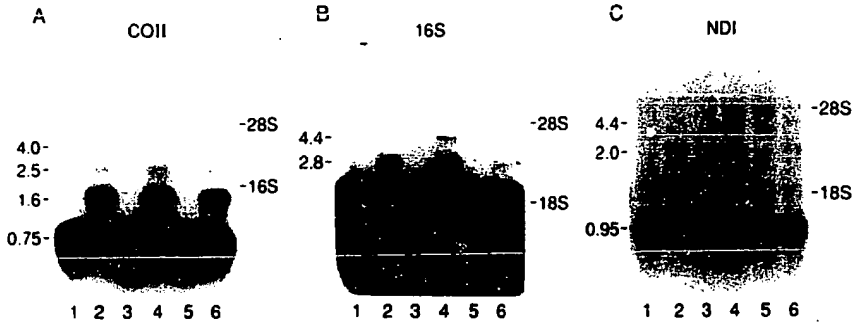


FIG. 3. **Northern blot detection of mitochondrial polycistronic RNA sequences in liver from 2-week HCCL-treated rats.** Total RNA was isolated from liver tissue obtained from control (*lanes 1, 3,* and *5*) and 2-week HCCL-treated (*lanes 2, 4,* and *6*) rats and analyzed by Northern analysis. Each lane corresponds to RNA (10 μg) from an individual rat (same rats as in Fig. 2). Migration of 18 and 28 S rRNAs are noted on the *right side* in each panel. Size estimates noted on the *left* of each panel are from separate gels incorporating full series of molecular weight markers. *A*, probe: CO II cDNA, 18-h exposure. *B*, probe: 16 S cDNA, 24-h exposure (mature transcript region overexposed). *C*, probe: ND1 cDNA, 5-day exposure. Note nonspecific hybridization to 18 and 28 S rRNAs in all lanes in *C*.

TABLE I

Hybridization of probes to polycistronic RNA species in HCCL-treated rats

Hepatic total RNA from 2-week HCCL-treated rats was prepared and probed with cDNAs or oligonucleotides for 12 and 16 S rRNA, ND1, CO I, CO II, ATPase 6, CYTb, and the light strand product in the CO II region (CO II-L). The presence of hybridization in all three animals tested at the molecular weights shown is indicated by a + and absence of hybridization by a −. The approximate mature transcript size for each gene is noted for reference. "Mature" indicates mature transcript present in this region.

	Mature transcript							
	12 S, 0.95 kb	16 S, 1.6 kb	ND1, 0.95 kb	CO I, 1.6 kb	CO II, 0.75 kb	ATPase 6, 0.85 kb	CYTb, 1.1 kb	CO II-L, none
RNA size (kb)								
1.6	−	Mature	−	Mature	+	+	−	−
2.5	−	−	−	−	+	+	−	−
2.8	+	+	+	−	−	−	−	−
4.0	−	−	−	−	+	−	−	−
4.4	+	+	+	−	−	−	−	−

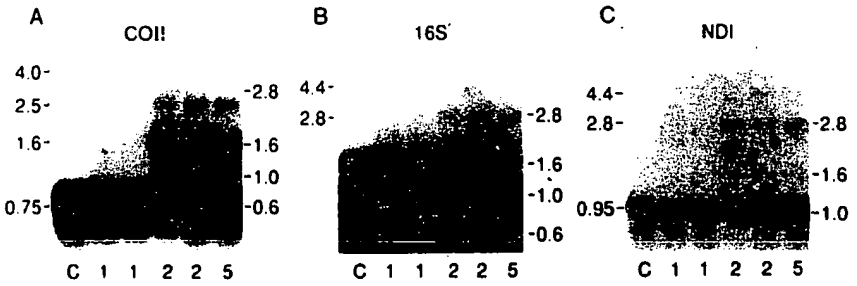


FIG. 4. **Time course of mitochondrial polycistronic RNA accumulation in HCCL-treated rats.** Total RNA was isolated from control (*c*), 1-week HCCL-treated (*1*), 2-week HCCL-treated (*2*), and 5-week HCCL-treated (*5*) rats and analyzed by Northern analysis. Each lane corresponds to 10 μg of RNA from an individual rat. Migration of size markers are noted on the *right* of each panel with size indicated in kilobases. Size estimates for bands are given on the *left side* of each panel. *A*, probe: CO II cDNA, 6-h exposure, *B*, probe: 16 S, 22-h exposure, C. Probe: ND1 cDNA, 9 day exposure.

ATPase 6 are contiguous (Fig. 1), and their sizes sum to 1.6 kb, it is probable that this represents an RNA combining the CO II-ATPase 6 mRNAs and intervening tRNAS (Fig. 1). Alternatively, two independent 1.6-kb RNAs, one containing CO II plus some unknown sequences, and one containing ATPase 6 plus some unknown sequence, cannot be definitively excluded using this approach. Analogously, a 2.5-kb RNA was identified by CO II and ATPase 6 probes, but not the CO I probe. A polycistronic sequence of the CO II, ATPase 6/8 and CO III regions would be predicted to be 2.5 kb and is consistent with the observed hybridization pattern (Fig. 1). The 12 S, 16 S, and ND1 probes all identified bands of 2.8 kb. The length of all three genes would exceed 2.8 kb, but the 12–16 S and 16 S ND1 sequences are each predicted to be 2.8 kb, and thus it is probable that both species are present (Fig. 1). A 4.4-kb RNA was identified by 12 S, 16 S, and ND1 probes and would be accounted for by a 12–16 S-ND1 RNA (Fig. 1). This reasoning suggests that the polycistronic mtRNAs accumulated with HCCL treatment contain contiguous uninterrupted mRNA and rRNA genes and are not the product of abnormal intragenic cleavages or splicing.

The HCCL-induced accumulation of the polycistronic mtRNAs described was estimated as 10–100-fold (data not shown). Increases in CO II containing polycistronic mtRNAs similar to those characterized in Fig. 2 have been reported in association with thyroid-induced mitochondrial proliferation (21), but in contrast to the HCCL model, thyroid treatment also increased mature mRNA content (9, 21). A 12–16 S polycistronic mtRNA has been reported in a hamster cell line treated with cycloleucine, an inhibitor of *S*-adenosylmethionine-dependent methylation (32). Similarly, the 16 S-ND1 mtRNA has been identified previously, and its content is elevated in tissues from patients with mtDNA mutations (33, 34).

HCCL treatment produces effects in liver indistinguishable from severe cobalamin deficiency, including decreased L-methylmalonyl-CoA mutase and methionine synthetase activities (12). Cobalamin deficiency decreases hepatic tetrahydrofolate

and *S*-adenosylmethionine contents (35, 36), which in turn may cause hypomethylation of nucleic acids (37, 38). Thus, HCCL treatment may interfere with mtRNA methylation required for normal nuclease processing. This concept is supported by the findings of Dubin *et al.* (39, 40), who have shown that mtRNA is methylated under physiologic conditions, and the reported accumulation of 12–16 S polycistronic mtRNA in cells treated with cycloleucine (32). Additionally, direct evidence exists for the role of substrate RNA methylation in RNA processing (41, 42).

Polycistronic mtRNAs containing CO II and ND1 coding sequences accumulated with HCCL treatment, whereas the mature mRNAs for these genes were decreased. Although HCCL-induced defective cleavage of precursor polycistronic mtRNA may cause a decrease in mtRNA content, CYTb mRNA content was also decreased and no CYTb-containing polycistronic mtRNAs were identified. Additionally, issues related to stability and turnover of the polycistronic mtRNAs make a cause-and-effect relationship difficult to establish. Decreased transcription beyond the potential 16 S premature termination site on the mtDNA (4) or accelerated mRNA degradation, both of which are potentially affected by nucleotide methylation, could also contribute to decreased mature mRNA contents.

The observed reduction in hepatic mitochondrial mRNA expression after 2 weeks of HCCL treatment suggests a potential mechanism for the electron transport chain defects that is manifested in liver mitochondria after 5 weeks of HCCL treatment (15). Mitochondrial content of cytochrome *b*, as well as activities of ubiquinol:cytochrome *c* oxidoreductase and cytochrome *c* oxidase activities per mg of mitochondrial protein are decreased (15). Decreased expression of the CYTb and CO II mitochondrial genes would explain these functional defects. The decrease in mitochondrial mRNAs observed when normalized per 28 S would be quantitatively more dramatic if expressed per mitochondrion (as for example estimated by citrate synthase activity). The appearance of the electron transport chain defect 3–4 weeks following a decrease in mRNA expression would also be consistent with the estimated 100+ h half-lifes of some electron transport chain proteins (43).

Alterations in mtDNA expression are now clearly documented to contribute to diverse disease pathologies (44, 45), and decreased mtDNA expression is a potential cause of neurologic and muscle disease (45). The HCCL-induced cobalamin-deficient rat represents a model of acquired defective mtDNA expression. The neurologic disease of human cobalamin deficiency is of unknown etiology, but is clearly related to altered methionine homeostasis (46). Based on the current studies, and consistent with the threshold model of mitochondrial disease described by Wallace (45), it is possible that altered mtDNA expression may contribute to the pathophysiology of cobalamin deficiency dementia. HCCL treatment provides a facile model for testing this relationship, and for studying the mechanisms of mtRNA expression and processing.

Acknowledgments—We thank Dr. Christine Van Itallie for providing the CO II construct and the 16 S and CYTb plasmids subcloned from original clones isolated by Dr. Howard Zalkin and Dr. Durwood Ray who provided the pKSU1 plasmid. We also thank William Vetter for technical assistance and Drs. C. L. Hoppel and J. Gott for constructive comments on the work described in this manuscript.

REFERENCES

1. Holloszy, J. O., and Coyle, E. F. (1984) *J. Appl. Physiol.* **56**, 831–838
2. Aw, T. Y., and Jones, D. P. (1989) *Annu. Rev. Nutr.* **9**, 229–251
3. Attardi, G., and Schatz, G. (1988) *Ann. Rev. Cell Biol.* **4**, 289–333
4. Clayton, D. A. (1991) *Annu. Rev. Cell Biol.* **7**, 453–478
5. Nelson, B. D. (1987) *Curr. Top. Bioenerg.* **15**, 221–272
6. Nagley, P. (1991) *Trends Genet.* **7**, 1–4
7. Chang, D. D., and Clayton, D. A. (1987) *Science* **235**, 1178–1184
8. Kadowaki, T., and Kitagawa, Y. (1988) *FEBS Lett.* **233**, 51–56
9. Mutvie, A., Kuzela, S., and Nelson, B. D. (1989) *Eur. J. Biochem.* **180**, 235–240
10. Coleman, W. B., and Cunningham, C. C. (1991) *Biochim. Biophys. Acta* **1058**, 178–186
11. Brass, E. P., Allen, R. H., Ruff, L. J., and Stabler, S. P. (1990) *Biochem. J.* **266**, 809–815
12. Stabler, S. P., Brass, E. P., Marcell, P. D., and Allen, R. H. (1991) *J. Clin. Invest.* **87**, 1422–1430
13. Krahenbuhl, S., Ray, D. B. Stabler, S. P., Allen, R. H., and Brass, E. P. (1990) *J. Clin. Invest.* **86**, 2054–2061
14. Tandler, B., Krahenbuhl, S., and Brass, E. P. (1991) *Anat. Rec.* **231**, 1–6
15. Krahenbuhl, S., Chang, M., Brass, E. P., and Hoppel, C. L. (1991) *J. Biol. Chem.* **266**, 20998–21003
16. Srere, P. A. (1969) *Methods Enzymol.* **13**, 3–11
17. Labarca, C., and Paigen, K. (1980) *Anal. Biochem.* **102**, 344–352
18. Chumczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
19. Herrin, D. L., and Schmidt, G. W. (1988) *BioTechniques* **6**, 196–200
20. Van Itallie, C. M., and Dannies, P. S. (1988) *Mol. Endocrinol.* **2**, 332–336
21. Van Itallie, C. M. (1990) *Endocrinology* **127**, 55–62
22. Gadaleta, G., Pepe, G., DeCandia, G., Quagliariello, C., Sbisa, E., and Saccone, C. (1989) *J. Mol. Evol.* **28**, 497–516
23. Bonnett, R., Cannon, J. R., Clark, V. M., Johnson, A. W., Parker, L. R., Smith, L. E., and Todd, A. (1957) *J. Chem. Soc.* 1158–1168
24. Dolphin, P. (1971) *Methods Enzymol.* **18**, 34–52
25. Herrera, E., and Freinkel, N. (1968) *Biochim. Biophys. Acta* **170**, 244–253
26. Mutvei, A., Kuzela, S., and Nelson, B. D. (1989) *Eur. J. Biochem.* **180**, 235–240
27. Williams, R. S., Garcia-Moll, M., Mellor, J., Salmons, S., and Harlan, W. (1987) *J. Biol. Chem.* **262**, 2764–2767
28. Canatore, P., Flagella, Z., Fracasso, F., Lezza, A. M. S., Gadaleta, M. N., and de Montalvo, A. (1987) *FEBS Lett.* **213**, 144–148
29. Gaines, G., Rossi, C., and Attardi, G. (1987) *J. Biol. Chem.* **262**, 1907–1915
30. Gelfand, R., and Attardi, G. (1981) *Mol. Cell. Biol.* **1**, 497–511
31. Brass, E. P. (1992) *Biochem. J.* **288**, 175–180
32. Prince, D. L., Kotin, R. M., and Dubin, D. T. (1986) *Mol. Biol. Rep.* **11**, 51–55
33. King, M. P., Koga, Y., Davidson, M., and Schon, E. A. (1992) *Mol. Cell. Biol.* **12**, 480–490
34. Heddi, A., Lestienne, P., Wallace, D. C., and Stepien, G. (1993) *J. Biol. Chem.* **268**, 12156–12163
35. Doi, T., Kanate, T., Tadano, T., Tijima, T., and Maekawa, A. (1989) *J. Nutr. Sci. Vitaminol.* **35**, 1–9
36. Wilson, S. D., and Horne, D. W. (1986) *Arch. Biochem. Biophys.* **244**, 248–253
37. Kliasheva, R. I. (1983) *Biol. Nauki. (Mosc.)* **10**, 17–19
38. Christman, J. K., Sheikhnejad, G. Dizik, M., Abileah, S., and Wainfan, E. (1993) *Carcinogenesis* **14**, 551–557
39. Dubin, D. T., and Taylor, R. H. (1978) *J. Mol. Biol.* **121**, 523–540
40. Dubin, D. T., Taylor, R. H., and Davenport, L. W. (1978) *Nucleic Acids Res.* **5**, 4385–4397
41. Ullu, E., and Tschudi, C. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10074–10078
42. Narayan, P., and Rottman, F. M. (1992) *Adv. Enzymol. Relat. Areas Mol. Biol.* **65**, 255–285
43. Hare, J. F., and Hodges, R. (1982) *J. Biol. Chem.* **257**, 3575–3580
44. Grossman, L. I. (1990) *Am. J. Hum. Genet.* **46**, 415–417
45. Wallace, D. C. (1992) *Annu. Rev. Biochem.* **61**, 1175–1212
46. Metz, J. (1992) *Annu. Rev. Nutr.* **12**, 59–79

INTER LIBRARY LOAN REQUEST FORM

Y.N05/30

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	07/654,116	Req. Date	ASAP	Date	

lete

110147313 CA: 110(17)147313c JOURNAL
Effect of cobalamin inactivation on folate metabolism of leukemic cells
AUTHOR(S): Ermens, Anton A. M.; Kroes, Alois C. M.; Schoester, Martijn; Van Lom, Kirsten; Lindemans, Jan; Abels, Johan
LOCATION: Inst. Hematol., Erasmus Univ., Rotterdam, Neth.
JOURNAL: Leuk. Res. DATE: 1988 VOLUME: 12 NUMBER: 11-12 PAGES: 905-10
CODEN: LEREDD ISSN: 0145-2126 LANGUAGE: English
SECTION:
CA201006 Pharmacology
IDENTIFIERS: nitrous oxide cobalamin folate metab leukemia, neoplasm inhibitor folate metab nitrous oxide
DESCRIPTORS:
Neoplasm inhibitors,leukemia...
cobalamin antagonist nitrous oxide enhancement of, in humans, folate metab. alteration in
CAS REGISTRY NUMBERS:
10024-97-2 biological studies, cobalamin inactivation by, folate metab. of human leukemic cells response to
59-30-3 biological studies, metab. of, by human leukemic cells, cobalamin inactivation by nitrous oxide effect on
51-21-8 52-52-8 59-05-2 folate metab. of human leukemic cells alteration by cobalamin inactivation by nitrous oxide and, neoplasm inhibition in relation to
365-07-1 formation of, by human leukemic cells, cobalamin inactivation by nitrous oxide effect on
13408-78-1 inactivation of, by nitrous oxide, folate metab. of human leukemic cells response to

JL68047

32166

3977-6

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

Leukemia Research Vol. 12, Nos. 11/12, pp. 905–910, 1988.
Printed in Great Britain.

0145-2126/88 $3.00 + .00
Pergamon Press plc

EFFECT OF COBALAMIN INACTIVATION ON FOLATE METABOLISM OF LEUKEMIC CELLS

ANTON A. M. ERMENS, ALOIS C. M. KROES, MARTIJN SCHOESTER, KIRSTEN VAN LOM, JAN LINDEMANS and JOHAN ABELS

Institute of Hematology, Erasmus University Rotterdam, The Netherlands

(*Received* 10 *June* 1988. *Revision accepted* 31 *July* 1988)

Abstract—Exposure to nitrous oxide inactivates the cobalamin coenzyme of methionine synthetase, an essential enzyme in folate metabolism. Hemopoietic cells are especially dependent on the function of cobalamin for the folate-dependent synthesis of thymidylate (dTMP). Inhibition of methionine synthetase may therefore be of potential value in the treatment of hematological malignancies. In the present study we investigated the effect of nitrous oxide induced cobalamin inactivation on folate metabolism of fresh leukemic cells and the human myelomonocytic cell line U937. Cells were exposed to nitrous oxide for 20 h. Subsequently they were subjected to the deoxyuridine suppression test (dU test), which measures the disturbance of folate-dependent dTMP synthesis. In all bone marrow samples, cobalamin inactivation resulted in a 200% increase of the dU test value, implicating a decreased *de-novo* synthesis of dTMP. Incubation of leukemic cells with methotrexate, 5-fluorouracil or cycloleucine induced similar increases of the dU test values which could be further raised to 400% with the addition of N_2O exposure. Prolonged experiments with U937 cells revealed that the disturbance of folate metabolism aggravated up to 48 h of nitrous oxide exposure. It can be concluded that cobalamin inactivation in human leukemic cells results in disturbed folate-dependent dTMP synthesis. Moreover, effects of several drugs interfering with folate metabolism can be enhanced.

Key words: Cobalamin, vitamin B_{12}, leukemia, folate, nitrous oxide.

INTRODUCTION

COBALAMIN, or vitamin B_{12}, is a nutritional factor indispensable for the maintenance of normal hematopoiesis. This is explained by its role as a coenzyme in methionine synthetase, or 5-methyltetrahydrofolate homocysteine methyltransferase, the enzyme which is essential for the intracellular conversion of reduced folates into forms that can be retained in the cell. Several steps in the biosynthesis of nucleotides require the presence of folate coenzymes. In particular the *de-novo* synthesis of thymidylate (dTMP) from uridylate (dUMP) appears to be sensitive to reduced availability of the necessary coenzyme 5,10-methylene tetrahydrofolate (see Fig. 1). As dTMP is an indispensable precursor for DNA synthesis, this provides the link between cobalamin deficiency and a disturbance of hematopoietic proliferation.

This immediate importance to proliferating cells makes the metabolism of reduced folates a potential target in the chemotherapy of malignant tumors. Structural analogues of reduced folates, notably methotrexate (MTX), have demonstrated the validity of this approach. Attempts have been made to find similar antagonists of the essential coenzyme function of cobalamin in methionine synthetase. However early efforts, mainly with structural analogues, were largely unsuccessful [1–3]. No effective antagonist of cobalamin was known until the recognition of a peculiar side effect of the anesthetic gas nitrous oxide (N_2O) in 1978 [4], which already was known to cause megaloblastic hematopoiesis on prolonged exposure [5]. N_2O inactivates the cobalamin coenzyme of methionine synthetase [7] by oxidation of its cobalt (I) moiety. This particular reaction results in a virtually complete and irreversible inhibition of this enzyme [6]. The availability of a genuine cobalamin antagonist has revived the interest in possible applications in chemotherapy.

In addition, the demonstration of the cobalamin dependence of malignant proliferation is of more

Abbreviations: N_2O, nitrous oxide; *MTX*, methotrexate; *5-FU*, 5-fluorouracil; *dU test*, deoxyuridine suppression test; *dUMP*, uridylate; *dTMP*, thymidylate; *AML*, acute myeloid leukemia; *ALL*, acute lymphoid leukemia; *BNML*, Brown Norway myeloid leukemia; *MDS*, myelodysplastic syndrome.

Correspondence to: Dr A. A. M. Ermens, Institute of Hematology, Ee2202 Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

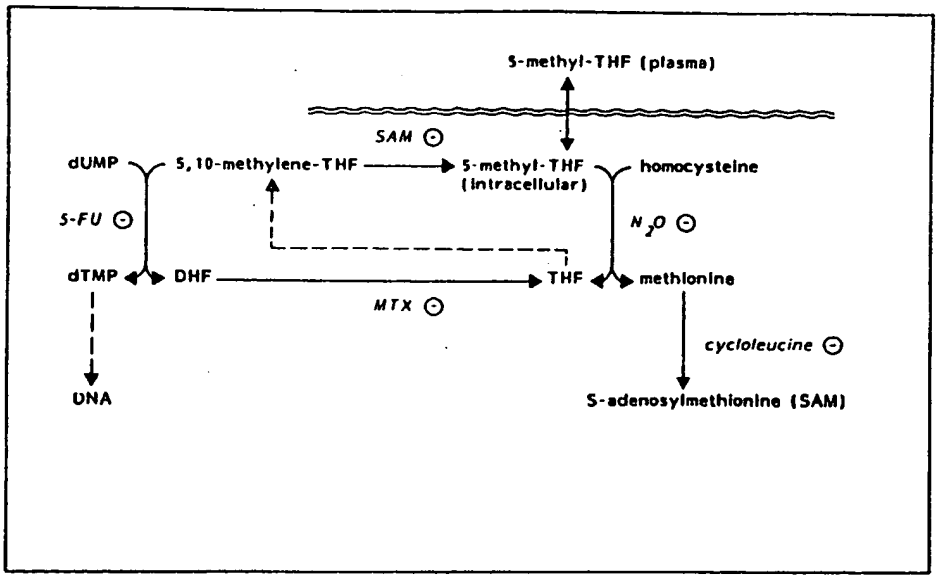


FIG. 1. Outline of the most important intracellular interconversions of reduced folates and the principal biochemical targets of the drugs used in this study.

general interest. We have shown previously that nitrous oxide reduces the growth of leukemia in rats [8], and renders the leukemic rats more susceptible to subsequent therapy of several drugs interfering with other pathways of folate metabolism (see Fig. 1) [9–11]. Kano *et al.* [12] have demonstrated that N_2O inhibits nucleotide synthesis and proliferation in a number of human hematopoietic cell lines.

In this study, we have investigated the *in-vitro* effects of N_2O on human leukemic cells derived from patients with different types of acute leukemia and the myelomonoblastic cell line U937 [13]. Moreover combination of cobalamin inactivation with MTX, 5-FU and cycloleucine, an effective inhibitor of methionine adenosyltransferase [14], was studied. The assessment of the sensitivity of human leukemia to N_2O should be considered an essential step towards the application of cobalamin inactivation in clinical chemotherapy. The deoxyuridine suppression test, being an established method to demonstrate the metabolic consequences of cobalamin deficiency or inactivation, was used to evaluate the effects of nitrous oxide.

MATERIALS AND METHODS

Leukemic bone marrow and blood cells

Bone marrow aspirates and peripheral blood samples were obtained from consenting patients with a diagnosis of acute leukemia. The patients were untreated, or, in some cases, in early relapse of their disease. Each marrow aspirate, usually 1–3 ml, was collected in a sterile bottle containing 0.4 ml (2000 IU) heparin. Peripheral blood was collected in heparinized tubes. Each case of acute myeloid leukemia (AML) was morphologically and cytochemically classified according to the FAB classification [15, 16], and cases of acute lymphoid leukemia (ALL) were classified according to immunological phenotype. The percentage of leukemic blasts in each bone marrow sample was determined in a differential count of 500 cells.

Isolation of nucleated cells from marrow and blood

Bone marrow aspirates and peripheral blood samples were diluted with an equal volume of Hanks' balanced salt solution and gently admixed with 0.5 ml methylcellulose solution (2% w/v) to promote rouleaux formation. After 10 min, the red cells were sedimented and nucleated cells could be isolated from the supernatant. The cells were washed in Hanks' balanced salt solution, counted and diluted to a concentration of 10^6/ml with McCoy's medium 5A with 10% fetal calf serum. Folic acid (pteroylglutamic acid) was replaced by 400 nmol/l methyltetrahydrofolate (Sigma, St Louis, U.S.A.), and methionine was limited to 25 μmol/l, as these constituents have to be carefully controlled in studies on cobalamin and folic acid metabolism. Viability of the cells was checked by trypan blue exclusion.

Culture of the cell line U937

The myelomonoblastic cell line was cultured in suspension, in specially prepared McCoy's medium 5A similar to the one described above. Experimental conditions were as described by Kano *et al.*, with 200 nmol/l of 5-methyltetrahydrofolate [10] and were carried out with cells in a logarithmic growth phase. Exposure to nitrous oxide and subsequent incubations were performed as described for bone marrow cells.

Exposure of cells to nitrous oxide

Cell suspensions (5 ml in flasks of 50 ml) were exposed to nitrous oxide by gassing the culture flasks during 30 min with a filtered mixture of 50% nitrous oxide, 20% oxygen, 25% nitrogen and 5% carbon dioxide, released from a premixed cylinder (Hoekloos, Schiedam, The Netherlands). After this period, the flasks were closed and incubated overnight for about 20 h at 37°C. Control suspensions were exposed to a mixture of air with 5% carbon dioxide, and otherwise treated identically. After incubation, viability was checked again by trypan blue exclusion and the cells were directly subjected to the dU test.

Addition of drugs

If enough cells were available, MTX (final concentration 5.10^{-8} mol/l, Ledertrexate), 5-FU (final concentration 5.10^{-6} mol/l, Hoffmann LaRoche) or cycloleucine (final concentration 10^{-3} mol/l, Sigma) were added to the cell suspensions at the start of the exposure to the gas mixtures and remained there during the whole incubation period.

Deoxyuridine suppression test

This test was used to evaluate the impairment of the *de-novo* synthesis of thymidylate as a consequence of the disturbance of folate metabolism. [^{3}H]-Thymidine incorporation into DNA is measured with and without the pre-incubation of cells with deoxyuridine. Deoxyuridine suppresses the incorporation of [^{3}H]-thymidine if it can be converted to thymidylate by folate-dependent methylation. If this conversion is impaired, the suppression by deoxyuridine will be reduced. The results of this assay are expressed as the percentage ratio of the [^{3}H]-thymidine incorporation with and without deoxyuridine in similar cell suspensions. The test was carried out essentially as described by Metz *et al.* [17], with some modifications. Briefly 10^6 cells in 1 ml Hanks' balanced salt solution with or without 0.1 mmol/l dU were incubated for 2 h at 37°C, followed by another 2-h incubation in the presence of 0.3 μCi [^{3}H]-thymidine (specific activity: 25 Ci/mmol, Amersham, U.K.). All results were the mean of triple incubations.

Statistical analysis

Data were statistically evaluated with the Wilcoxon's signed-ranks test for two groups.

RESULTS

Patient characteristics

Eighteen patients were involved in this study and their characteristics are summarized in Table 1. The mean blast count of the blood samples was 63 ± 22%, the bone marrow cell fractions contained 70 ± 25% leukemic blasts.

Effect of cobalamin inactivation on fresh leukemic samples

The results of deoxyuridine suppression tests after nitrous oxide exposure of leukemic marrow cells, compared to the same cells exposed to air, are shown in Table 2. In all cases, the exposure to nitrous oxide caused a marked increase of the test value, indicating an impaired ability to use dUMP as a substrate in the *de-novo* synthesis of dTMP. In normal healthy bone marrow the percentage suppression by dU varies but usually the upper level of the normal range is considered to be 10%. The variation of suppression values after exposure to air, from 3 to 15% in our study, with a mean of 9%, is probably related to the heterogeneity of cell types in different forms of leukemia and the patients physical condition. DU tests of leukemic bone marrow performed directly after the aspiration show a similar degree of variation (data not shown). After nitrous oxide exposure of cells derived from the same bone marrow samples, the test values range from 8 to 57%, with a mean of 21% (significance: $p < 0.01$). Results obtained with blood cells from nine leukemia patients, are also presented in Table 2. The increase in mean suppression values, from 8 to 14%, is comparable to the result obtained with the marrow cells. However this time the pattern is somewhat less consistent as two of the nine samples were found to be unresponsive to nitrous oxide, both from cases of ALL.

TABLE 1. CHARACTERISTICS OF LEUKEMIA PATIENT SAMPLES USED FOR *IN-VITRO* COBALAMIN INACTIVATION

Number of patients involved	18
Male	11
Female	7
Diagnosis	
Acute myeloid leukemia	12
Acute lymphoid leukemia	4
Leukemic transformed M.D.S.	2
Studied material	
Bone marrow aspirates	16
Peripheral blood	9

Effect of N_2O with concomitant exposure to other drugs

Methotrexate was added, prior to the incubation, to six of the leukemic marrow cell suspensions and two peripheral blood cell suspensions. The results are

TABLE 2. EFFECT OF N_2O EXPOSURE (20 h) ON THE dU SUPPRESSION VALUE OF LEUKEMIC CELLS DERIVED FROM BONE MARROW AND PERIPHERAL BLOOD (mean ± S.D.)

		dU test values	
Origin of cells	*n*	Air	N_2O
Bone marrow	16	9.0 ± 3.8%	21.4 ± 13.2%
Peripheral blood	9	7.5 ± 4.5%	13.4 ± 8.1%

TABLE 3. EFFECT OF SEVERAL DRUGS AND THE CONCOMITANT EXPOSURE TO N_2O (20 h) ON THE dU SUPPRESSION VALUE OF LEUKEMIC BONE MARROW CELLS (mean ± S.D.)

n	Air	N_2O	MTX (5.10^{-8} M)	N_2O + MTX
6	10.5 ± 3.0%	22.0 ± 10.1%	38.9 ± 17.2%	49.5 ± 12.2%
n	Air	N_2O	5-FU (5.10^{-6} M)	N_2O + 5-FU
7	7.4 ± 4.2%	21.1 ± 15.8%	34.7 ± 22.1%	46.3 ± 22.6%
n	Air	N_2O	cycloleucine (10^{-3} M)	N_2O + cycloleucine
5	7.9 ± 3.7%	20.5 ± 4.7%	9.5 ± 2.1%	27.9 ± 4.2%

shown in Tables 3 and 4 respectively. The addition of methotrexate alone to the marrow cells increased the suppression value in all cases, from a mean of 11% to a mean value of 39%. If the cells were, in addition, exposed to N_2O, this resulted in a significant, further increase of the mean suppression value to 50% ($p < 0.05$). In some cases, however, in which methotrexate was already very effective, no substantial further increase was observed; this applies to values of 60% and higher. It should be emphasized that these values indicate a rather extreme disturbance of the folate metabolism. Therefore it is possible that aggravation of this disturbance by cobalamin inactivation is not detectable anymore. The two peripheral samples, demonstrated a similar enhanced effect of methotrexate after concomitant exposure to N_2O.

A comparable pattern was obtained with the combination of N_2O with 5-FU (see Tables 3 and 4). Both blood samples and six of the seven marrow samples showed an appreciable effect of 5-FU on the dU test value (mean 35%), which could be significantly aggravated by N_2O (mean 46%, $p < 0.05$).

Cycloleucine by itself did not affect the dU suppression value in most of the tested cases (mean 10%). In combination with N_2O however a sharp increase was noted (mean 27%) in all bone marrow samples ($p < 0.05$) and the two peripheral blood samples (see Tables 3 and 4).

Effects on the cell line U937

Finally, results obtained with the cell line U937, with culture conditions as described by Kano *et al.* [12], are presented in Table 5. Prolonged exposure to N_2O with or without 5-FU or MTX up to two days resulted in a time-dependent increase of the dU suppression values. Cells exposed to the drugs in combination with N_2O consistently showed the largest increase of the dU test value.

TABLE 4. EFFECT OF SEVERAL DRUGS AND THE CONCOMITANT EXPOSURE TO N_2O ON THE dU SUPPRESSION VALUE OF INDIVIDUAL LEUKEMIC CELL SAMPLES DERIVED FROM PERIPHERAL BLOOD

Air	N_2O	MTX	N_2O + MTX
8.6	8.5	24.4	29.5
15.5	25.2	52.5	68.5
Air	N_2O	5-FU	N_2O + 5-FU
6.2	13.4	15.7	29.6
13.5	21.5	25.2	36.4
Air	N_2O	cycloleucine	N_2O + cyclo
7.1	13.6	5.5	27.4
9.2	11.0	9.2	15.1

DISCUSSION

At present, there is only limited evidence for the cobalamin-dependence of malignant hematopoiesis in human leukemia, which is an essential requirement for the possible application of cobalamin antagonists in chemotherapy. N_2O, the only available effective

TABLE 5. EFFECT OF MTX OR 5-FU AND THE CONCOMITANT EXPOSURE TO N_2O FOR TWO DAYS ON THE dU SUPPRESSION VALUE OF U937 CELLS

Treatment	Day 0	Day 1	Day 2
Air	12.1	14.2	12.6
N_2O	12.1	16.3	24.2
MTX (5.10^{-8} M)	12.1	18.4	50.5
N_2O + MTX	12.1	24.7	77.4
Air	5.8	13.7	12.6
N_2O	5.8	12.1	11.1
5-FU (5.10^{-6} M)	5.8	23.2	34.7
N_2O + 5-FU	5.8	28.4	51.6

antagonist of the cobalamin coenzyme in methionine synthetase, has been shown to inhibit rat leukemia *in vivo* [8], and human leukemic cell lines *in vitro* [12]. There are also two early observations of a distinct antileukemic effect of nitrous oxide exposure in patients [18, 19] dating from the time before this agent was known to inactivate cobalamin. Moreover, two cases have been described of patients with leukemia, in which the administration of cobalamin was considered to have enhanced leukemic proliferation [20, 21].

A disturbance of the deoxyuridine suppression test in normal bone marrow cells after N_2O exposure of about 24 h is well established [22]. The results of the present study provide evidence that in nearly all cases of acute leukemia the folate metabolism can also be disturbed by cobalamin inactivation, as is apparent from elevated values of the deoxyuridine suppression test. This *in-vitro* metabolic effect of cobalamin inactivation on malignant blood cells is fully comparable to the consequences of cobalamin deficiency on normal marrow cells. It may therefore be expected that the application of nitrous oxide will result in the same inhibition of cellular proliferation as is observed in cobalamin deficient normal marrow cells.

Combination of N_2O with other drugs resulted in an enhanced disturbance of the folate metabolism. These observations are in accordance with a study in which the effect of N_2O with MTX on normal bone marrow cells was examined [23]. More interestingly our observations agree well with studies of the antileukemic activity of N_2O combined with respectively cycloleucine, MTX, and 5-FU in the BNML. In this rat leukemia, which is considered to be a reliable model for human AML, the effective antiproliferative action of the three combinations mentioned above, was also reflected by severely disturbed dU test values [8–11].

As nearly all cases of leukemia were responsive to cobalamin inactivation, it is difficult to draw conclusions on the differential sensitivity of particular types. It may be relevant, however, that all samples that were found to be unresponsive to N_2O were cases of ALL.

With regard to the results obtained with the leukemic cell line U937, it can be concluded that the effects of cobalamin inactivation in combination with other drugs increase over substantial periods of time. This may possibly reflect the gradual changes in folate coenzyme pools induced by N_2O [24, 25], 5-FU [26] or MTX [27].

In conclusion, the results of this study demonstrate that the metabolic consequences of cobalamin inactivation by nitrous oxide can be observed in malignant hematopoietic cells, as in normal marrow cells. The deoxyuridine suppression test may well be predictive for the clinical sensitivity of acute leukemias to N_2O, as results obtained with N_2O *in vitro* are in agreement with those obtained by treating rat leukemia *in vivo*. Moreover, these findings encourage further investigations, eventually directed at the administration of N_2O to selected patients with leukemia.

REFERENCES

1. Lester Smith E. (1960) Biological activities of anti-vitamin B12 substances. *Acta haemat.* **24**, 9.
2. Csanyi E., Kelemen M. A. & Borsy J. (1962) Changes in the blood of the rat induced by the monocarboxylic acid of cyanocobalamin obtained by fermentation (anti-vitamin B12). *Nature, Lond.* **194**, 689.
3. Siddons R. C. (1974) Vitamin B12 antagonism by monocarboxylic acids and anilides of cyanocobalamin. *Nature, Lond.* **247**, 308.
4. Amess J. A. L., Burman J. F., Rees G. M., Nancekievill D. G. & Mollin D. L. (1978) Megaloblastic haemopoiesis in patients receiving nitrous oxide. *Lancet* **2**, 339.
5. Lassen H. C. A., Henriksen E., Neukirch F. & Kristensen H. S. (1956) Treatment of tetanus. Severe bone marrow depression after prolonged nitrous-oxide anaesthesia. *Lancet* **1**, 527.
6. Deacon R., Lumb M., Perry J. *et al.* (1980) Inactivation of methionine synthetase by nitrous oxide. *Eur. J. Biochem.* **104**, 419.
7. Banks R. G. S., Henderson R. J. & Pratt J. M. (1968) Reactions of gases in solution. Part III. Some reactions of nitrous oxide with transition-metal complexes. *J. chem. Soc.* 2886.
8. Kroes A. C. M., Lindemans J., Hagenbeek A. & Abels J. (1984) Nitrous oxide reduces growth of experimental rat leukemia. *Leukemia Res.* **8**, 441.
9. Kroes A. C. M., Lindemans J., Schoester M. & Abels J. (1986) Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (vitamin B12) by nitrous oxide. *Cancer Chemother. Pharmac.* **17**, 114.
10. Kroes A. C. M., Lindemans J. & Abels J. (1986) Synergistic growth inhibiting effect of nitrous oxide and cycloleucine in experimental rat leukaemia. *Br. J. Cancer* **50**, 793.
11. Kroes A. C. M., Ermens A. A. M., Lindemans J. & Abels J. (1986) Effects of 5-fluorouracil treatment on rat leukemia with concomitant inactivation of cobalamin. *Anticancer Res.* **6**, 737.
12. Kano Y., Sakamoto S., Sakuraya K. *et al.* (1983) Effects of nitrous oxide on human cell lines. *Cancer Res.* **43**, 1493.
13. Sundstrom C. & Nilsson K. (1976) Establishment and characterization of a human histiocytic lymphoma cell line (U-937). *Int. J. Cancer* **17**, 565.
14. Lombardini J. B., Coulter A. W. & Talalay P. (1970) Analogues of methionine as substrates and inhibitors of the methionine adenosyl-transferase reaction. *Molec. Pharmac.* **6**, 481.
15. Bennett J. M., Catovsky D., Daniel M. T., *et al.* (1976)

Proposals for the classification of the acute leukaemias. *Br. J. Haemat.* **33,** 451.
16. Bennett J. M., Catovsky D., Daniel M. T. *et al.* (1985) Proposed revised criteria for the classification of acute myeloid leukemia. *Ann. intern. Med.* **103,** 620.
17. Metz J., Kelly A., Swett V. C., Waxman S. & Herbert V. (1968) Deranged DNA synthesis by bone marrow from vitamin B12-deficient humans. *Br. J. Haemat.* **14,** 575.
18. Lassen H. C. A. & Kristensen H. S. (1959) Remission in chronic myeloid leukaemia following prolonged nitrous oxide inhalation. *Dan. med. Bull.* **6,** 252.
19. Eastwood D. W., Green C. D., Lambdin M. A. & Gardner R. (1964) Effect of nitrous oxide on the white-cell count in leukemia. *New Engl. J. Med.* **268,** 297.
20. Corcino J. J., Zalusky R., Greenberg M. & Herbert V. (1971) Coexistence of pernicious anaemia and chronic myeloid leukaemia: an experiment of nature involving vitamin B12 metabolism. *Br. J. Haemat.* **20,** 511.
21. Ahmann F. R. & Durie B. G. M. (1984) Acute myelogenous leukaemia modulated by B12 deficiency: a case with bone marrow blast cell corroboration. *Br. J. Haemat.* **58,** 91.
22. Skacel P. O., Hewlett A. M., Lewis J. D., Lumb M., Nunn J. F. & Chanarin I. Studies on the haemopoietic toxicity of nitrous oxide in man. *Br. J. Haemat.* **53,** 189.
23. Kano Y., Sakamoto S., Sakuraya K. *et al.* (1981) Effect of nitrous oxide on human bone marrow cells and its synergistic effect with methionine and methotrexate on functional folate deficiency. *Cancer Res.* **41,** 4698.
24. Wilson S. D. & Horne D. W. (1986) Effect of nitrous oxide inactivation of vitamin B12 on the levels of folate coenzymes in rat bone marrow, kidney brain and liver. *Archs Biochem. Biophys.* **244,** 248.
25. Lumb M., Perry J., Deacon R. & Chanarin I. (1981) Changes in tissue folates accompanying nitrous oxide-induced inactivation of vitamin B12 in the rat. *Am. J. clin. Nutr.* **34,** 2412.
26. Houghton J. A., Schmidt C. & Houghton P. J. (1982) The effect of derivatives of folic acid on the fluorodeoxyuridylate-thymidilate synthetase covalent complex in human colon xenografts. *Eur. J. Cancer Clin. Oncol.* **18,** 347.
27. Baram J., Allegra C. J., Fine R. L. & Chabner B. A. (1987) Effect of methotrexate on the intracellular folate pools in purified myeloid precursor cells from normal human bone marrow. *J. clin. Invest.* **79,** 692.

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

nplete

02732162 BIOSIS NO.: 000068042761
POSSIBILITY OF INCREASING THE ANTI NEOPLASTIC ACTION OF A FOLIC-ACID **ANTAGONIST** BY METHYL **COBALAMIN** ANALOGS
AUTHOR: SOF'INA Z P; MYASISHCHEVA N V; ARSENYAN F G; YURKEVICH A M
AUTHOR ADDRESS: ONCOL. SCI. CENT., ACAD. MED. SCI. USSR, MOSCOW, USSR.
JOURNAL: VESTN AKAD MED NAUK SSSR (1). 1979. 72-78. 1979
FULL JOURNAL NAME: Vestnik Akademii Meditsinskikh Nauk Sssr
CODEN: VAMNA
RECORD TYPE: Abstract
LANGUAGE: RUSSIAN

419866

JK. 1105/30

TC68053

ABSTRACT: The effect of methylcobalamine and its analog (difluorochloromethylcobalamine and methylcobalamine chloropalladate) on the growth of transplantable tumors in mice [adenocarcinoma of the mammary gland (Ca-755), carcinoma of the uterine cervix (CUC-5) and carcinoma of the intestine (ACATOL)] was studied. The activity of the cobalamine coenzyme analogs was investigated when used alone or combined with inhibitors of dihydrofolate reductase and methionine synthetase. The results of the experiments indicate a stimulating effect of methylcobalamine on the growth of transplantable solid tumors in the animals. The antitumor activity of the methylcobalamine analog studied was higher in combined application with methotrexate. The most effective inhibition of tumor growth and longest survival of animals were achieved in combined application of methylcobalamine with methotrexate and methionine synthetase inhibitor, depending on the scheme of administration.

39777 4

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

УДК 615.277.3:577.164.161

З. П. СОФЬИНА, Н. В. МЯСИЩЕВА, Ф. Г. АРСЕНЯН, А. М. ЮРКЕВИЧ

ВОЗМОЖНОСТЬ УСИЛЕНИЯ ПРОТИВООПУХОЛЕВОГО ДЕЙСТВИЯ АНТАГОНИСТА ФОЛИЕВОЙ КИСЛОТЫ АНАЛОГАМИ МЕТИЛКОБАЛАМИНА

Онкологический научный центр АМН СССР, Москва

Стимулирующее действие цианокобаламина на рост перевиваемых опухолей разного вида животных (саркома кур Рауса, фибросаркома PW-2, саркома 45 и ССК крыс, карцинома Герена, саркома 180 и лимфосаркома мышей) и ослабление лечебного действия некоторых противоопухолевых препаратов при совместном их применении с витамином B_{12}, отмечаемые в ранних исследованиях, обусловлены активным биосинтезом его коферментов в организме животных. Оценка функциональной роли метилкобаламина — одного из кобаламиновых коферментов в процессах роста нормальных и опухолевых клеток — привлекает наибольшее внимание.

Метилкобаламин является коферментом метионинсинтетазной реакции — ключевого звена, определяющего синергизм действия кобаламинов и соединений фолиевой кислоты в процессах клеточной пролиферации. Особая значимость метилкобаламина для активации этой ферментной системы отмечена в результате изучения нарушенного обмена кобаламинов при лейкозах человека. Малая эффективность комбинированной цитостатической терапии при определенных вариантах острого лейкоза, протекающих с высокой концентрацией метилкобаламина в крови, подтверждала специфичность его действия в организме (Н. В. Мясищева и соавт., 1969). В настоящее время установлена активная роль метилкобаламина в процессах пролиферации клеток кроветворной ткани здоровых животных. Под воздействием метилкобаламина в селезенке мышей возрастают число клеток, синтезирующих ДНК, их митотическая активность и величина пролиферативного пула (О. Д. Голенко и соавт.). Обнаружено значительное увеличение частоты развития гемобластозов у мышей при комбинированном введении метилкобаламина с эндогенными бластомогенами. Важным моментом механизма стимулирующего действия кобаламинов является их индуцирующее влияние на активность метионинсинтетазы. В культурах нормальных клеток млекопитающих и опухолевых клеток человека активность метионинсинтетазы заметно возрастает с увеличением содержания кобаламинов в среде культивирования (Mangum и соавт.; Kamely и соавт.). Опухолевые клетки разного типа, однако, отличны от нормальных по своей способности под воздействием кобаламинов усиливать биосинтез метионина, необходимый при интенсивном росте (Halpern и соавт.; Chello и Bertino). Спасательный путь с помощью кобаламинзависимой метионинсинтетазы, обеспечивая увеличение внутриклеточного пула тетрагидрофолиевой кислоты независимо от фолатредуктазной системы, представляет, по-видимому, основной механизм развития устойчивости лейкозных клеток к метотрексату (МТХ) (Н. В. Мясищева; Sauer и Jaenicke).

В связи с этим реальна возможность усиления противоопухолевого эффекта данного метаболита путем его комбинированного применения

УДК 615.277.3:577.164.161

АРСЕНЯН, А. М. ЮРКЕВИЧ

ОТИВООПУХОЛЕВОГО ОЙ КИСЛОТЫ АНАЛОГАМИ ИНА

МН СССР, Москва

іамина на рост перевиваемых ма кур Рауса, фибросаркома а Герена, саркома 180 и лим- ого действия некоторых проти- і их применении с витамином , обусловлены активным био- ивотных. Оценка функциональ- кобаламиновых коферментов вых клеток — привлекает наи-

ом метионинсинтетазной реак- инергизм действия кобалами- оцессах клеточной пролифера- для активации этой фермент- чения нарушенного обмена ая эффективность комбиниро- еделенных вариантах острого нтрацией метилкобаламина в его действия в организме стоящее время установлена сах пролиферации клеток кро- од воздействием метилкобал- исло клеток, синтезирующих ичина пролиферативного пула ачительное увеличение часто- и комбинированном введении могенами. Важным моментом баламинов является их инду- нсинтетазы. В культурах нор- левых клеток человека актив- тает с увеличением содержа- ия (Mangum и соавт.; Kamely ипа, однако, отличны от нор- ействием кобаламинов усили- при интенсивном росте (Hal- ельный путь с помощью ко- еспечивая увеличение внутри- лоты независимо от фолатре- мому, основной механизм раз- ок к метотрексату (МТХ)

усиления противоопухолевого омбинированного применения

с антагонистами кобаламинового кофермента. Понимание механизма действия кобаламинов послужило обоснованием для направленного синтеза аналогов метилкобаламина и их испытания в качестве потенциальных противоопухолевых соединений.

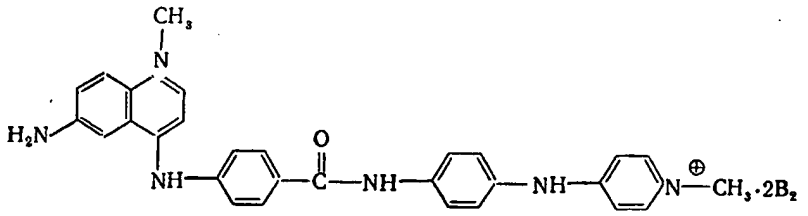
В химиотерапевтических экспериментах были изучены дифторхлорметилкобаламин и хлорпаллодат метилкобаламина, обнаруживавшие активность при исследованиях in vitro в подавлении роста бактериальных клеток и торможении синтеза ДНК в культуре эмбриональных фибробластов человека (Н. В. Мясищева и соавт., 1977).

При разработке схемы комбинированного воздействия были учтены основные аспекты физиологического действия кобаламинов в организме: контроль за поступлением соединений фолиевой кислоты в клетки и образованием коферментов фолата, а также интенсивность поглощения кобаламинов опухолевыми клетками (Burke и соавт.; Tisman и Herbert; Floodh и Ullberg). В связи с этим можно было рассчитывать на избирательность действия исследуемых соединений и возможность снижения активности кобаламинозависимого фермента в организме. Однако трудно было ожидать значительного эффекта при их изолированном применении. Поэтому нам представлялось важным оценить противоопухолевое действие этих соединений на фоне торможения активности дигидрофолатредуктазы с помощью МТХ.

Материал и методы. Исследования проведены на мышах линии $C_{57}BL$, CBA, BALB/c и гибридах $BDF_1/C_{57}BL \times DBA(2)$, массой 20—25 г, полученных из питомника АМН СССР. Противоопухолевая активность аналогов метилкобаламина изучена на перевиваемых лейкозах L-1210 и La и солидных опухолях: аденокарциноме молочной железы (Са-755), раке шейки матки (РШМ-5) и аденокарциноме кишечника (АКАТОЛ). Мы выбрали в качестве основного объекта исследования солидные опухоли, на которых легче выявить стимулирующее влияние метилкобаламина, чем на модели лейкозов мышей L-1210 и La, с высоким пролиферативным пулом и весьма короткой продолжительностью жизни животных.

Метилкобаламин (CH_3Cbl) и дифторхлорметилкобаламин (CF_2ClCbl) получены по известному методу (Wood и соавт., 1968), модифицированному в разделе выделения (Е. М. Тачкова и соавт.). Хлорпаллодат метилкобаламина ($MetCbl \cdot PdCl_3$) синтезирован способом Е. Г. Чаусера. Метилкобаламин вводили внутримышечно из расчета 10 мкг/кг 2 раза на курс лечения с интервалом 96 ч, CF_2ClCbl — ежедневно подкожно из расчета 500 мг/кг одномоментно либо 2 раза в день по 250 мг/кг в течение 5 дней. Плохо растворимый хлорпаллодат метилкобаламина вводили перорально в 2% крахмальной суспензии в суточной дозе 500 мг/кг в течение 5 дней или 2 раза с интервалом 96 ч. Суточная доза вводилась одномоментно или по 250 мг 2 раза в день. МТХ фирмы «Lederle» использовали в дозе 10 мг/кг внутрибрюшинно с интервалом 96 ч.

В наших исследованиях активность кобаламиновых производных изучена не только при комбинированном применении с МТХ, но также с хинолиновым производным (NSC-176319):



Препарат получен нами из Национального института рака США в соответствии с соглашением о сотрудничестве между СССР и США в области химиотерапии опухолей. Согласно характеристике, представленной американскими учеными, препарат является ингибитором метионинсинтетазы (Carter и соавт.). Хинолиновое производное применяли внутрибрюшинно в дозе 5 мг/кг ежедневно или с интервалом 96 ч, что составляет половину максимально переносимой дозы для использованного режима. Лечение начинали через 48 ч после перевивки опухоли. Результаты воздействия оценивали через 24 ч после окончания курса лечения и в различные сроки на протяжении жизни животных. Критерием эффективности служили процент торможения роста опухоли, вычисляемой по условному объему, и увеличение продолжительности жизни животных. В каждом из опытов контрольные и опытные группы составляли так, чтобы их численность обеспечивала статистическую значимость минимальных учитываемых процентов торможения

роста опухолей (50%) и увеличения продолжительности жизни мышей (25%). В соответствии с указанными требованиями опытные группы состояли из 6—10 мышей, а контрольные — из 6—13 животных, в зависимости от используемого штамма опухоли.

Результаты и их обсуждение. В проведенных исследованиях впервые обнаружено стимулирующее влияние метилкобаламина на рост перевиваемых опухолей Са-755, АКАТОЛ, в меньшей степени — на рост РШМ-5 (табл. 1). Наибольшая интенсивность роста опухоли под воздействием метилкобаламина наблюдалась при перевивке Са-755 мышам-гибридам BDF_1 (180%) по сравнению с ростом той же опухоли у мышей чистой линии $C_{57}Bl$. Стимуляция размножения опухолевых клеток происходила в период введения метилкобаламина; наибольшее различие в величине опухолей у животных опытной и контрольной групп выявлено непосредственно после окончания введения препарата. В последующие сроки рост опухолей у мышей, получавших метилкобаламин, замедлялся. При перевивке АКАТОЛ мышам разного пола интенсивность роста опухоли при воздействии метилкобаламина различна. Стимулирующее действие препарата было значительнее выражено у самцов (см. табл. 1).

Как и следовало ожидать, изолированное воздействие аналогов метилкобаламинов тормозило рост перевиваемых опухолей Са-755, РШМ-5 в небольшой степени и лишь непосредственно после введения препаратов (табл. 2).

При сравнительной оценке наибольшая ингибирующая активность обнаружена при использовании хлорпаллодоата метилкобаламина. Эффективность торможения роста Са-755 была более выражена у мышей-гибридов BDF_1 по сравнению с мышами $C_{57}Bl$. Как было указано, именно у мышей BDF_1 в значительно большей степени проявлялось и стимулирующее действие метилкобаламина. В этой серии опытов продолжительность жизни мышей BDF_1 с аденокарциномой молочной железы при воздействии CF_2ClCbl и хлорпаллодата метилкобаламина увеличивалась на 50% (см. табл. 2). В то же время при введении производных метилкобаламина отсутствовал эффект торможения роста АКАТОЛ. Отмечено большое различие в действии кобаламиновых производных на опухоль в зависимости от режима их применения (см. табл. 2). По-видимому, при однократном введении большой дозы (500 мг/кг) возможна диссоциация препаратов с последующим образованием активной формы, стимулирующей рост опухоли.

В соответствии с нашим предположением при комбинировании аналогов метилкобаламина с МТХ обнаружено усиление их действия на опухоль (Са-755, РШМ-5; табл. 3). Увеличение противоопухолевого эффекта в результате комбинированного воздействия проявлялось непосредственно после курса введения препаратов и, особенно, в последующий период: когда эффект действия одного МТХ уже отсутствовал, сохранялся достаточно высокий процент торможения роста опухоли.

Таблица 1

Влияние метилкобаламина на рост некоторых перевиваемых опухолей

Опухоль	Доза препарата, мкг/кг	Срок введения препарата после прививки опухоли, дни	Рост опухоли после введения препарата, % к контролю		
			1 день	7 дней	14 дней
Са-755 $C_{57}BL$	10	2-й и 6-й	+74	+21	+23
BDF_1	10	2-й и 6-й	+180	+65	+10
АКАТОЛ:	10	2-й и 6-й	+20	+23	+31
самки					
самцы	10	2-й и 6-й	+126	+37	+33

Примечание. Здесь и в табл. 2—6 знак «плюс» обозначает стимуляцию роста опухоли.

ельности жизни мышей (25%). В со- группы состояли из 6—10 мышей, а и от используемого штамма опухоли.

и е. В проведенных исследова- щее влияние метилкобаламина АКАТОЛ, в меньшей степени — я интенсивность роста опухоли людалась при перевивке Са-755 нению с ростом той же опухоли яция размножения опухолевых метилкобаламина; наибольшее ных опытной и контрольной окончания введения препарата. у мышей, получавших метил- АКАТОЛ мышам разного пола ействии метилкобаламина раз- ата было значительнее выраже-

анное воздействие аналогов ме- евиваемых опухолей Са-755, епосредственно после введения

шая ингибирующая активность лодоата метилкобаламина. Эф- ыла более выражена у мышей- $C_{57}Bl$. Как было указано, имен- й степени проявлялось и стиму- этой серии опытов продолжи- карциномой молочной железы ата метилкобаламина увеличи- емя при введении производных торможения роста АКАТОЛ. кобаламиновых производных применения (см. табл. 2). По- ольшой дозы (500 мг/кг) воз- ующим образованием активной

нием при комбинировании ана- кено усиление их действия на ичение противоопухолевого эф- воздействия проявлялось непо- ратов и, особенно, в последую- дного МТХ уже отсутствовал, торможения роста опухоли.

Таблица 1

орых перевиваемых опухолей

препара- вки опу- и	Рост опухоли после введения препарата, % к контролю		
	1 день	7 дней	14 дней
-й	+74	+21	+23
-й	+180	+65	+10
-й	+20	+23	+31
-й	+126	+37	+33

обозначает стимуляцию роста опухоли.

Таблица 2

Противоопухолевое действие аналогов метилкобаламина

Опухоль	Препарат	Доза препаратов, мкг/кг	Срок введения препаратов после прививки опухоли, дни	Торможение роста опухоли, % к контролю			Увеличение продолжительности жизни мышей, % к контролю
				1 день	7 дней	15 дней	
Са-755	Хлордифторметилкобаламин (CF_2ClCbl)	250+250	2—6-й	30	+8		54
		250+250	2—6-й	43	38	0	16
РШМ-5		250+250	2—6-й	0	0	0	0
АКАТОЛ							
Са-755	Комплекс трихлорметилкобаламина с ($MetCb \cdot PdCl_3$)	250+250	2—6-й	90	59		50
(BDF_1)		500	2—6-й	13	16	20	
		250+250	2—6-й	80	23	0	10
РШМ-5		500	2—6-й	+130	+15	+18	0
АКАТОЛ		250+250	2—6-й	0	0	0	0

Для понимания возможного механизма действия аналогов метилкобаламина в организме животных был осуществлен сравнительный анализ роста тех же опухолевых штаммов при изолированном влиянии ингибитора метионинсинтетазы — хинолинового производного — и его сочетанного воздействия с МТХ. Торможение роста Са-755, РШМ-5 и АКАТОЛ увеличивалось в зависимости от концентрации препарата. Наиболее эффективно препарат воздействовал на Са-755. При увеличении дозы от 5 до 15 мг/кг торможение роста опухоли возрастало соответственно до 40 и 96%. Однако с увеличением дозы препарата заметно возрастала и его токсичность. Например, при штаммах лейкозов L-1210 и La наиболее оптимальной дозой, по нашим данным, являлась доза 10 мг/кг, при которой в 3—4 раза увеличивалась продолжительность жизни животных. При уменьшении дозы эффект воздействия препарата на мышей с лейкозами был существенно ниже. При солидных опухолях в наших исследованиях не было отмечено значительного увеличения продолжительности жизни мышей. При сочетанном введении препарата с МТХ даже в малой дозе (5 мг/кг) наблюдалась суммация эффекта, что подтверждало увеличение торможения роста опухоли (табл. 4). При более позднем начале лечения животных (на 8-й день после перевивки опухоли) и ежедневном введении препаратов в течение 5 сут (5 мг/кг хинолинового производного; 2 мг/кг МТХ) результаты были еще более демонстративны (Са-755), но при суммарном воздействии увеличивалась также и общая токсичность (табл. 5).

Увеличение торможения роста опухоли и продолжительности жизни животных отмечено при комбинированном воздействии хлорпаллодата метилкобаламина и хинолинового производного (NSC-176319; табл. 6). Учитывая усиление действия МТХ при его комбинированном использовании с аналогами метилкобаламина и ингибитором метионинсинтетазы, мы осуществили комбинированное лечение мышей с Са-755 с применением всех 3 ингибиторов: МТХ, хинолинового производного и наиболее активного аналога кобаламинового кофермента — хлорпалладата метилкобаламина (см. табл. 6).

В результате комбинированного применения ингибиторов метионинсинтетазы и дигидрофолатредуктазы значительно усиливалось противоопухолевое действие, особенно в отдаленные сроки после окончания лечения. В этих условиях через 2 нед после окончания введения препаратов торможение роста опухоли составляло 85%, в то время как в группах мышей, получавших каждое из исследуемых соединений изолированно или комбинацию из 2 препаратов, в эти сроки угнетение роста

Таблица 3

Противоопухолевое действие комбинации МТХ и аналогов метилкобаламина

Опухоль	Препарат	Доза введения препаратов, м г/кг	Срок введения препаратов после прививки, дни	Торможение роста опухоли, % к контролю					Увеличение продолжительности жизни мышей, % к контролю
				1 день	5 дней	7 дней	10 дней	14 дней	
Са-755 ($C_{57}BL$)	МТХ	10	2-й и 6-й	75		10	+32		16
	$MetCbl \cdot PdCl_3$	250+250	2-й и 6-й	58		20	14		0
	МТХ+$MetCbl \cdot PdCl_3$	10+250+250 (вводились одновременно)	2—6-й	97		75	0		0
РШМ-5 (СВА)	МТХ	10	2-й и 6-й	90		48		40	0
	$MetCbl \cdot PdCl_3$	500	2-й и 6-й	+220		+100		+80	0
	МТХ+$MetCbl \cdot PdCl_3$	10+500 (вводились одновременно)	2-й и 6-й	97		65		40	40
Са-755 (гибриды)	МТХ	10	2-й и 6-й	87	81	45	67		
	CF_2ClCbl	500	2-й и 6-й	+67	+5	+21	5		
	МТХ+CF_2ClCbl	10+500 (CF_2ClCbl, вводился за 3 ч до МТХ)	2-й и 6-й	97	99	74	67		

Таблица 4

Действие комбинированного применения МТХ и NSC-176319 на опухоли мышей

Опухоль	Препарат	Доза препаратов, мг/кг	Срок введения препарата после прививки опухоли, дни	Торможение роста опухолей, % к контролю				
				1 день	5 дней	7—8 дней	10 дней	14—16 дней
Са-755 (BDF_1)	МТХ	5	2-й и 6-й	46	9	19	19	23
	NSC-176319	5	2-й и 6-й	18	8	41	29	30
	МТХ+NSC-176319	5+5 (вводились одновременно)	2-й и 6-й	81	62	66	43	31
РШМ-5 (СВА)	МТХ	10	2-й и 6-й	69		74		65
	NSC-176319	10	2-й и 6-й	20		55		31
	МТХ+NSC-176319	10+10 (вводились одновременно)	2-й и 6-й	88		84		75
АКАТОЛ (BALB/c)	МТХ	10	2-й и 6-й	45	53	44		
	NSC-176319	5	2-й и 6-й	12	27	30		
	МТХ+NSC-176319	10+5 (МТХ вводился через 20 мин после NSC-176319)	2-й и 6-й	65	43	40		

Опухоль	Препарат	мг/кг	Срок введения препарата после прививки опухоли, дни	1 день	5 дней	7—8 дней	10 дней	14—16 дней
Са-755 (BDF_1)	MTX	5	2-й и 6-й	46	9	19	19	23
	NSC-176319	5	2-й и 6-й	18	8	41	29	30
	MTX+NSC-176319	5+5 (вводились одновременно)	2-й и 6-й	81	62	66	43	31
РШМ-5 (СВА)	MTX	10	2-й и 6-й	69		74		65
	NSC-176319	10	2-й и 6-й	20		55		31
	MTX+NSC-176319	10+10 (вводились одновременно)	2-й и 6-й	88		84		75
АКАТОЛ (BALB/c)	MTX	10	2-й и 6-й	45	53	44		
	NSC-176319	5	2-й и 6-й	12	27	30		
	MTX+NSC-176319	10+5 (MTX вводился через 20 мин после NSC-176319)	2-й и 6-й	65	43	40		

Таблица 5

Действие MTX и NSC-176319, применяющихся в комплексе, на рост Са-755 мышей

Препарат	Доза препаратов мг/кг	Срок введения препарата после прививки опухоли, дни	Торможение роста опухоли, % к контролю		Отношение числа погибших животных к числу животных в группе
			1 день	3 дня	
MTX	2	8—12	12	+13	1/6
NSC-176319	5	8—12	12	+8	0/6
MTX+NSC-176319	2+5 (вводились одновременно)	8—12	76	79	5/6

Таблица 6

Действие на рост Са-755 мышей MTX, NSC-176319 и комплекса трихлорметилкобаламина с палладием

Препарат	Доза препаратов, мг/кг	Срок введения препаратов после прививки опухоли, дни	Торможение роста опухоли, % к контролю			Увеличение продолжительности жизни, % к контролю
			2 дня	8 дней	14 дней	
MTX	10	2-й и 6-й	99	51	0	14
NSC-176319	5	2-й и 6-й	37	7	+29	0
$MetCbl \cdot PdCl_3$	250	2-й и 6-й	75	40	13	0
NSC-176319+$MetCbl \cdot PdCl_3$	5+250 (вводились одновременно)	2-й и 6-й	90	58	4	23
$MetCbl \cdot PdCl_3$+MTX	250+10 (MTX вводился через 20 мин после $MetCbl \cdot PdCl_3$)	2-й и 6-й	99	58	5	8
NSC-176319+MTX	5+10 (MTX вводился через 20 мин после NSC-176319)	2-й и 6-й	99	88	44	0
NSC-176319+$MetCal \cdot PdCl_3$+MTX	5+250+10 (NSC-176319 и $MetCbl \cdot PdCl_3$ вводились одновременно, а MTX-через 20 мин после них)	2-й и 6-й	00 1	95	85	20

опухолей практически отсутствовало. Однако следует отметить, что одновременно повышалась токсичность. Установлено также, что действие комбинации препаратов существенно изменялось в зависимости от последовательности введения комбинантов и интервалов между ними. Так, одновременное введение NSC-176319 и МТХ оказалось значительно менее токсичным для организма, чем введение их с интервалом 3 ч при равном противоопухолевом эффекте.

Таким образом, результаты экспериментальных исследований подтверждают наше предположение о возможности усиления противоопухолевого действия МТХ с помощью аналогов метилкобаламина и ингибитора метионинсинтетазы. Это открывает новый подход к лечебному воздействию на опухоли с использованием антагонистов физиологического регулятора обмена соединений фолиевой кислоты в организме. Нами установлена противоопухолевая активность антагонистов кобаламинового кофермента. Однако активность исследованных аналогов метилкобаламина, блокирующих определенные метаболические звенья, недостаточна высока для полного и длительного торможения роста опухоли. Противоопухолевое действие аналогов кобаламинового кофермента может быть значительно усилено путем их комбинированного применения с МТХ. Полученные экспериментальные данные указывают на целесообразность испытания эффективности аналогичного рода комбинаций в клинике. Наша основная задача в настоящее время состоит в разработке оптимального режима комбинированного лечения опухолей указанными препаратами на основе всестороннего анализа механизма их сочетанного действия в организме.

ЛИТЕРАТУРА

Голенко О. Д., Мясищева Н. В., Раушенбах М. О. и др. — Вопр. мед. химии, 1974, № 5, с. 549—554. — Мясищева Н. В. Характеристика обмена соединений В-12 (кобаламинов) при лейкозах. Автореф. дис. докт. М., 1972. — Мясищева Н. В., Левина Г. Д., Лорие Ю. И. и др. — Пробл. гематол., 1969, № 4, с. 20—25. — Мясищева Н. В., Голенко О. Д., Кузнецова Л. Е. и др. — Вопр. мед. химии, 1977, № 5, с. 622—628. — Тачкова Е. М., Рудакова И. П., Мясищева Н. В. и др. — Биоорганич. химия, 1976, № 4, с. 535—541. — Burke G. T., Mangum J. H., Brodie J. D. — Biochemistry (Wash.), 1971, v. 10, p. 3079—3085. — Chello P. I., Bertino J. R. — Biochem. Pharmacol., 1975, v. 25, p. 889—892. — Floodh H., Ullberg S. — Int. J. Cancer, 1968, v. 3, p. 694—699. — Halpern B. C., Clark B. R., Hardy D. N. et al. — Proc. nat. Acad. Sci. USA, 1974, v. 71, p. 1133—1136. — Kamely D., Littlefield J. W., Erbe R. W. — Ibid., 1973, v. 70, p. 2585—2589. — Mangum J. H., Byron K., Murray J. et al. — Biochemistry (Wash.), 1969, v. 8, p. 3496—3499. — Sauer H., Jaenicke L. — Blut, 1974, Bd 28, S. 321—327. — Tisman G., Herbert V. — Blood, 1973, v. 41, p. 465—469. — Wood Y. N., Kennedy T. S., Wolfe R. S. — Biochemistry (Wash.), 1968, v. 7, p. 1707—1713.

POSSIBILITY OF POTENTIATING THE ANTINEOPLASTIC ACTION OF FOLIC ACID ANTAGONIST BY METHYLCOBALAMINE ANALOGUES

Z. P. Sofyina, N. V. Myasischeva, F. G. Arsenyan, A. M. Yurkevich

Summary. The effect of methylcobalamine and its analogues (difluoro-chloromethylcobalamine — CF_2ClCbl and methylcobalamine chloropalladate — $MetCbl \cdot DdCl_3$) on the growth of transplantable tumours in mice: adenocarcinoma of the mammary gland (Ca-755), carcinoma of the uterine cervix (CUC-5), carcinoma of the intestine (ACATOL) was studied. The activity of the cobalamine coenzyme analogues was investigated when used alone or combined with inhibitors of dehydrofolate reductase and methyonine synthetase. The results of the experiments indicate a stimulating effect of methylcobalamine on the growth of transplantable solid tumours in the animal organism. The antitumour activity of the methylcobalamine analogues studied was found to be higher in combined application with methotrexate. The most effective inhibition of tumour growth and the longer survival of the animals were achieved in combined application of methylcobalamine with methotrexate and methyonine synthetase inhibitor, depending upon the scheme of administration.

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Takahata et al.
Journal/Book Title:	J. Nutr. Sci Vitaminol.
Article Title:	
Volume (Issue):	41(5)
Pages:	515-526
Year of Publication:	1995
Publisher:	
Remarks:	

7068054 11/05/30

8750203

STIC Use Only Accession Number: 3977821

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

J. Nutr. Sci. Vitaminol., 41, 515–526, 1995

Synthesis, Properties and Microbiological Activity of Hydrophobic Derivatives of Vitamin B_{12}

Yusuke TAKAHATA, Ari NISHIZAWA, Ichiro KOJIMA,[1]
Mamoru YAMANISHI, and Tetsuo TORAYA*

Department of Biotechnology, Faculty of Engineering, Okayama University, Tsushima-Naka, Okayama 700, Japan
[1]*Central Technical Research Laboratory, Nippon Oil Company, Ltd., 8, Chidori-cho, Naka-ku, Yokohama 231, Japan*

(Received June 1, 1995)

Summary Long chain alkylcobalamins and long chain acyl-cyanocobalamins, two types of hydrophobic derivatives of vitamin B_{12}, were synthesized. It was shown by TLC and determination of the partition coefficient between organic and aqueous phases that the hydrophobicity of alkylcobalamins and acyl-cyanocobalamins increased with the chain length of the alkyl or acyl group introduced into cobalamin. Long chain alkylcobalamins were easily converted to aquacobalamin by photoirradiation, but the first-order rate constant of photolysis decreased with the length of an alkyl group. Long chain acyl-cyanocobalamins were gradually hydrolyzed to cyanocobalamin in neutral or alkaline solution with the pseudo-first order rate constant increasing with the pH of the solution. Stabilization of acyl-cyanocobalamins toward hydrolysis was achieved by introducing a methyl group into the α-position of an acyl group. All the long chain alkylcobalamins tested supported the growth of *Escherichia coli* 215, a cobalamin- or L-methionine-auxotroph, and *Lactobacillus leichmannii*, although their activity as cobalamin was at most 28% and 15% that of cyanocobalamin for *E. coli* 215 and *L. leichmannii*, respectively.
Key Words vitamin B_{12}, alkylcobalamin, acyl-cyanocobalamin, hydrophobic B_{12} derivatives, microbiological activity.

Vitamin B_{12} or Cbl is an essential micronutrient for humans. When taken up into living cells, it is converted into the two coenzyme forms, AdoCbl and MeCbl, and participates as coenzymes in several enzymatic reactions. This vitamin was first

* To whom correspondence should be addressed.

Abbreviations: Cbl, cobalamin; AdoCbl, adenosylcobalamin or coenzyme B_{12}; MeCbl, methylcobalamin; CN-Cbl, cyanocobalamin or vitamin B_{12}; EtCbl, ethylcobalamin; aqCbl, aquacobalamin; Cbl^{II}, cob(II)alamin or B_{12r}; Cbl^{I}, cob(I)alamin or B_{12s}; DMSO, dimethyl sulfoxide; DMF, *N,N*-dimethylformamide.

isolated as an anti-pernicious anemia factor in the liver (*1,2*). It has been also established that intrinsic factor in gastric juice is required for its intestinal absorption (*3*). The complex of the vitamin with this glycoprotein traverses the small bowel to the distal ileum where Cbl is absorbed into the intestinal cells through the ileal receptor for intrinsic factor-Cbl (*4,5*). Cbl bound to transcobalamin II is taken up by the cells through the receptor for transcobalamin II (*6*).

Malabsorption of cobalamin leads to pernicious anemia or other metabolic disorders caused by cobalamin deficiency. Congenital intrinsic factor deficiency is known to be the most common cause of cobalamin malabsorption (*7*).

For such patients, it would be very beneficial if easily absorbable derivatives of Cbl were available which could be incorporated into the cells not by the normal absorption and transport systems but by passive diffusion. These derivatives may become effective therapeutic agents. Hydrophobic Cbl derivatives are possible candidates for such easily absorbable derivatives. In addition, it is desirable that hydrophobic groups introduced into Cbl are severed from these derivatives under physiological conditions forming nontoxic compounds. In the present paper, we designed and synthesized two types of hydrophobic derivatives of Cbl, alkyl-Cbl and acyl-CN-Cbl, which can be back converted into Cbl itself in living organisms. Some of their properties and microbiological activity is reported here.

EXPERIMENTAL PROCEDURES

Chemicals. 2-Methylbutyric anhydride was synthesized by reaction of 2-methylbutyryl chloride with pyridinium 2-methylbutyrate. 2-Methylbutyryl chloride was synthesized by reaction of 2-methylbutyric acid with thionyl chloride. CN-Cbl was obtained from Glaxo Research Laboratories, Greenford, UK. All other chemicals were reagent grade commercial products and were used without further purification.

Analytical procedures. Purity and hydrophobicity of alkyl-Cbl and acyl-CN-Cbl synthesized were evaluated by TLC on silica gel. The solvent systems used are (A) water-saturated 2-butanol; and (B) 1-butanol/2-propanol/water (10 : 7 : 10, v/v). Paper electrophoresis was performed in 0.5 M acetic acid (pH 2.7) or in 10 mM potassium phosphate buffer (pH 7.0). The concentration of Cbl was determined spectrophotometrically after conversion to dicyano-Cbl. Alkyl-Cbl were converted to dicyano-Cbl by photolysis in the presence of 0.1 M KCN. ε_{367} of $30.4 \times 10^3\,M^{-1}cm^{-1}$ for dicyano-Cbl (*8*) was used throughout. UV and visible spectra were measured on a Union SM-401 or a Milton Roy SP-3000 recording spectrometer. Positive-ion fast atom bombardment-mass spectrometry (FAB-MS) was carried out on a VG-70SE mass spectrometer.

Synthesis and purification of octadecyl-Cbl and other hydrophobic alkyl-Cbl. CN-Cbl (50 mg, 37 μmol) in 5 ml of water was reduced with 150 mg (4.0 mmol) of $NaBH_4$. After 15 min at room temperature, 125 mg (0.37 mmol) of octadecyl bromide in 5 ml of ethanol was added in the dark. The reaction was terminated 90

min later by adding 5 ml of 30% (v/v) acetone. The reaction mixture was diluted with 50 ml of water and the product octadecyl-Cbl was extracted with 30 ml of 1-butanol. The 1-butanol extraction was repeated twice. After washing with 10–20 ml of water three times, the 1-butanol layer pooled was evaporated to dryness. The residue was suspended in 20 ml of water and applied to carboxymethylcellulose (H^+ form). After washing the column with water, the product was eluted with 1 M NaCl in 30% ethanol. Octadecyl-Cbl desalted by re-extraction with 1-butanol after adjusting the pH to 7 was confirmed to be homogeneous by TLC and stored at −20°C as a solution in 30% ethanol.

Other hydrophobic alkyl-Cbl, such as dodecyl-, octyl- and hexyl-Cbl, were synthesized, desalted and purified by similar procedures. EtCbl was desalted conventionally by extraction with phenol instead of 1-butanol because of its low hydrophobicity.

Synthesis of dodecanoyl-CN-Cbl and other hydrophobic acyl-CN-Cbl. Dodecanoic anhydride (0.61 g, 1.6 mmol) was melted by warming up to 42°C. CN-Cbl (20 mg, 15 μmol) in 5 ml of a mixture of dry DMSO and DMF (1 : 1, v/v) was added, and the reaction mixture was kept at 42°C with vigorous stirring. After 23 h, the reaction was terminated by adding 10 ml of diethyl ether and 10 ml of water in this order. The product was extracted by adding 40 ml of diethyl ether and 40 ml of water to the reaction mixture. The aqueous layer was washed three times with 20 ml of diethyl ether, concentrated to a small volume, and applied to a column of XAD-2. After washing the column with water, the product was eluted with 50% 2-methyl-2-propanol. The Cbl-containing fractions were collected and evaporated to dryness. The desired product was further purified to homogeneity by paper chromatography in solvent B and HPLC on a reverse-phase (ODS) column (Cosmosil $5C_{18}$, Nacalai Tesque, Japan) using 62% methanol as mobile phase and stored at −80°C as a solution in 30% ethanol.

Other hydrophobic acyl-CN-Cbl were synthesized and purified by the same procedures except for the reaction temperature (72°C for octadecanoyl-, 38°C for decanoyl-, room temperature for octanoyl- and propionyl-CN-Cbl) and the methanol concentration of the mobile phase in HPLC (78% for octadecanoyl-, 55% for decanoyl-, 53% for octanoyl-, and 25% for propionyl-CN-Cbl). Pentanoyl-, 2-methylbutyryl- and 2,2-dimethylpropionyl-CN-Cbl were also synthesized by reaction of CN-Cbl with the corresponding acid anhydrides in DMSO at room temperature for 1, 5 and 3 days, respectively.

Alternative methods of purification of hydrophobic acyl-CN-Cbl. Since the above-mentioned purification procedure is laborious and is not suitable for large scale preparation, a brief method of purification was also developed. Hydrophobic acyl-CN-Cbl desalted by XAD-2 column chromatography as described above was purified by preparative TLC on silica gel in solvent A, desorbed from silica gel with 1-decanol and then precipitated by adding diethyl ether to the 1-decanol layer. The purity of dodecanoyl-CN-Cbl thus obtained was more than 95%.

Pentanoyl-CN-Cbl was purified by column chromatography on ODS (YMC

Co., Ltd., Japan) using 50% methanol. 2-Methylbutyryl- and 2,2-dimethylpropionyl-CN-Cbl were purified by preparative TLC on silica gel using solvent A, followed by column chromatography on ODS.

Determination of partition coefficients between organic and aqueous phases. Equal volumes of 1-butanol or 1-decanol and water were added to a small amount of the synthesized alkyl-Cbl or acyl-CN-Cbl. After mixing vigorously with a Vortex for 1 min, the mixture was allowed to stand for 3 h. Concentrations of each Cbl in the organic and aqueous phases were then determined spectrophotometrically from the absorbance at 500–525 nm for alkyl-Cbl or 550 nm for acyl-CN-Cbl. 1-Butanol, 1-decanol, chloroform, ethyl acetate and hexane were used as organic solvents.

Rates of hydrolytic conversion of acyl-CN-Cbl into CN-Cbl. Acyl-CN-Cbl was allowed to stand at 37°C in buffers of various pH. A small aliquot was withdrawn at an appropriate time intervals and applied to a column of ODS. After washing the column with water, the desalted Cbl-containing fraction was obtained by eluting with 100% methanol. The ratio of CN-Cbl formed to acyl-CN-Cbl remaining was analyzed by HPLC on the ODS column using 30% methanol as the mobile phase. Pseudo-first order rate constants (k) for hydrolysis of propionyl-CN-Cbl into CN-Cbl were determined at various pH by graphic analyses.

Determination of biological activity. Microbiological activity as Cbl was determined by the growth-supporting effects of long chain alkyl-Cbl for *Escherichia coli* 215, a Cbl- or L-methionine-auxotroph (*9*), and *Lactobacillus leichmannii* (*10*). The assay media and procedures were the same as those described previously (*11*), except that long chain alkyl-Cbl in 70% ethanol were added to the assay media without sterilization to a final ethanol concentration of 0.7% (v/v). An EC_{50} value was defined as a concentration of CN-Cbl or each derivative which gives the half-maximum growth of the test organisms. The maximum growth was obtained at a CN-Cbl concentration more than 1 nM for both *E. coli* 215 and *L. leichmannii*.

RESULTS AND DISCUSSION

Confirmation of the synthesis of long chain alkyl-Cbl

Long chain alkyl-Cbl purified to homogeneity showed absorption spectra quite similar to that of MeCbl (data not shown). Upon acidification, the color of the synthetic compounds turned to yellow, and their spectra became identical with that of the base-off form of MeCbl. As expected, the relative mobility of octyl-Cbl to CN-Cbl upon paper electrophoresis at pH 2.7 was essentially the same as that of aqCbl. However, the relative mobility of octadecyl-Cbl was much smaller than expected, probably because of its high hydrophobicity. The FAB-mass spectrum of dodecyl-Cbl (MW 1,497.8) exhibited a peak of quasimolecular ion (MH^+) at *m/e* 1499 (Fig. 1A). The prominent peak at *m/e* 1330 was assigned to the fragment ion ($MH^+ - C_{12}H_{25}$). The same fragment ion peak ($MH^+ - CN$) was observed at *m/e* 1331 with authentic CN-Cbl (MW 1,355.4) (Fig. 1C). The peaks observed at *m/e* 1069 and 971 were also detected with CN-Cbl. Thus, these peaks can be assigned

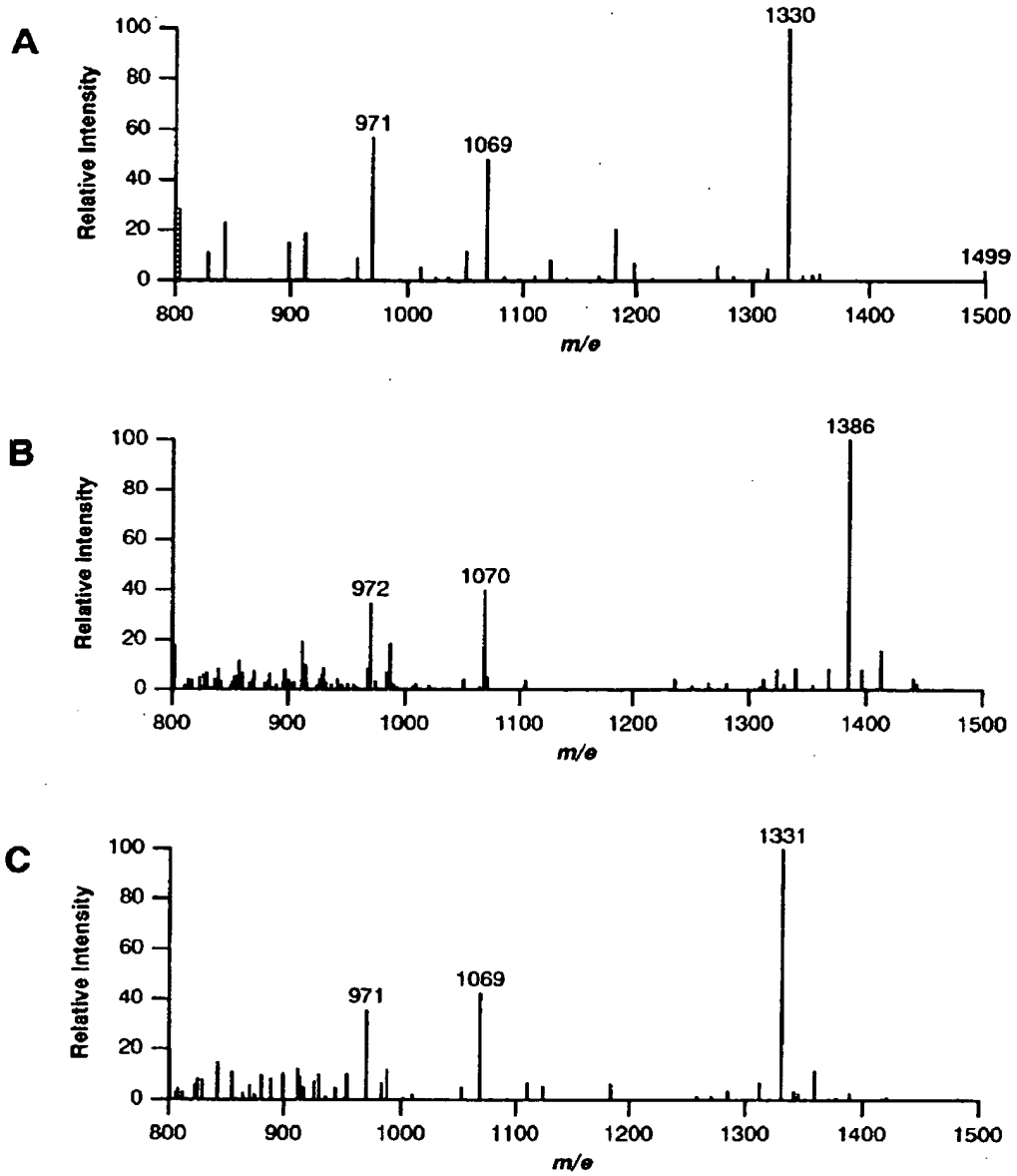


Fig. 1. FAB-mass spectra of dodecyl-Cbl (A), propionyl-CN-Cbl (B) and CN-Cbl (C).

to the fragment ions ($MH^+ - C_{12}H_{25} - \alpha$-ribazol-3′-yl) and ($MH^+ - C_{12}H_{25} - \alpha$-ribazole-3′-phosphate), respectively. From these results, it was concluded that the compound analyzed by FAB-MS is certainly dodecyl-Cbl. Threrfore, other compounds synthesized and purified by the same method can be considered to be the right compounds.

Confirmation of the synthesis of acyl-CN-Cbl

Long chain acyl-CN-Cbl purified to homogeneity showed absorption spectra identical to that of CN-Cbl (data not shown). The FAB-mass spectrum of propionyl-CN-Cbl (MW 1,411.5) did not provide the molecular ion or quasimolecular ion peak (Fig. 1B). However, the prominent peak appeared at *m/e* 1386 can be assigned to the fragment ion formed by removal of CN from quasimolecular ion ($MH^+ - CN$). The peaks at *m/e* 1070 and 972 were observed in common at *m/e* 1069 and 971 with authentic CN-Cbl (Fig. 1C) and dodecyl-Cbl (Fig. 1A). Therefore, it was concluded that this compound is certainly propionyl-CN-Cbl. It is evident that monoacylated CN-Cbl is the major product formed under the reaction conditions. In the synthesis of long chain acyl-CN-Cbl, some hydrophobic impurities were formed which were also back converted to CN-Cbl. As reported previously for succinylation of CN-Cbl (*12*), there are two sites to be acylated in CN-Cbl—namely, 5′-OH and 2′-OH of the ribose moiety of the nucleotide loop. From the difference of primary and secondary alcohols in reactivity toward acid anhydride, it is likely that the major product is the $O^{5'}$-monoacylated and one of the minor products is $O^{2'}$-acyl-CN-Cbl, although the location of the acyl group was not determined yet.

Hydrophobicity of long chain alkyl-Cbl and acyl-CN-Cbl

The hydrophobicity of the alkyl-Cbl and acyl-CN-Cbl synthesized was evaluated by TLC on silica gel in two solvent systems. As shown in Table 1, R_f values of the derivatives become higher with increasing number of carbon atoms in an alkyl or acyl group introduced into Cbl.

Hydrophobicity was also evaluated by determination of partition coefficients of alkyl-Cbl and acyl-CN-Cbl between organic and aqueous phases. As shown in

Table 1. R_f Values of alkyl-Cbl and acyl-CN-Cbl in TLC on silica gel.

Run No.	Cbl	R_f	
		Solvent A	Solvent B
1	CN-Cbl	0.08	0.19
	MeCbl	0.14	0.32
	EtCbl	0.15	0.36
	Hexyl-Cbl	0.18	0.41
	Octyl-Cbl	0.20	0.42
	Dodecyl-Cbl	0.21	0.43
	Octadecyl-Cbl	0.23	0.44
2	CN-Cbl	0.06	0.22
	Propionyl-CN-Cbl	0.07	0.26
	Octanoyl-CN-Cbl	0.17	0.43
	Decanoyl-CN-Cbl	0.17	0.44
	Dodecanoyl-CN-Cbl	0.18	0.45
	Octadecanoyl-CN-Cbl	0.20	0.46

Table 2. Partition coefficients of alkyl-Cbl and acyl-CN-Cbl between organic and aqueous phases.

Run No.	Cbl	Partition coefficient	
		1-Butanol/water	1-Decanol/water
1	MeCbl	0.25	0.00
	EtCbl	0.35	0.00
	Hexyl-Cbl	1.6	0.01
	Octyl-Cbl	4.2	0.07
	Dodecyl-Cbl	16	6.0
	Octadecyl-Cbl	25	7.0
2	Propionyl-CN-Cbl	0.09	0.00
	Octanoyl-CN-Cbl	6.1	0.23
	Decanoyl-CN-Cbl	28	0.91
	Dodecanoyl-CN-Cbl	31	2.5
	Octadecanoyl-CN-Cbl	58	7.5

Table 2, dodecyl-Cbl and octadecyl-Cbl, long chain homologs, were distributed chiefly into both the 1-butanol and 1-decanol phases rather than the aqueous phase, while MeCbl, EtCbl, short chain homologs, were distributed chiefly into the aqueous phase. Middle chain homologs, such as hexyl-Cbl and octyl-Cbl, were distributed chiefly into the organic layer in the 1-butanol/water system and in the aqueous layer in the 1-decanol/water system. Similar results were obtained with acyl-CN-Cbl homologs (Table 2). Therefore, it was concluded that increasing hydrophobicity of alkyl-Cbl and acyl-CN-Cbl was obtained by increasing length of an alkyl or acyl group introduced into Cbl. When chloroform, ethyl acetate or hexane was used as an organic solvent, essentially no distribution into an organic phase was observed.

A strong correlation has been reported between cellular uptake of porphyrins in V79 Chinese hamster cells and their distribution into octanol (*13*). Hence, the hydrophobic derivatives of Cbl reported in this paper may be taken up into cells by passive diffusion.

Conversion of long chain alkyl-Cbl to aqCbl

Like MeCbl and EtCbl (*14,15*), long chain alkyl-Cbl in 30% ethanol underwent photolytic cleavage of the Co-C bond forming aqCbl. Upon photolysis, spectra of alkyl-Cbl changed to that of aqCbl with several isosbestic points. This result suggests that alkyl-Cbl are almost directly converted to aqCbl—that is, Cbl^{II}, an intermediate in the photolytic process (*16*), is very short-lived. The rate of photolysis of alkyl-Cbl obeyed first-order kinetics. The effect of chain length on the first-order rate constant (k) is shown in Fig. 2. Except for MeCbl, the rate constant decreased with chain length of an alkyl group. This can be explained by assuming recombination of Cbl^{II} with an alkyl radical formed. Longer chain alkyl radicals may be kept near the place of formation by hydrophobic interaction with the corrin

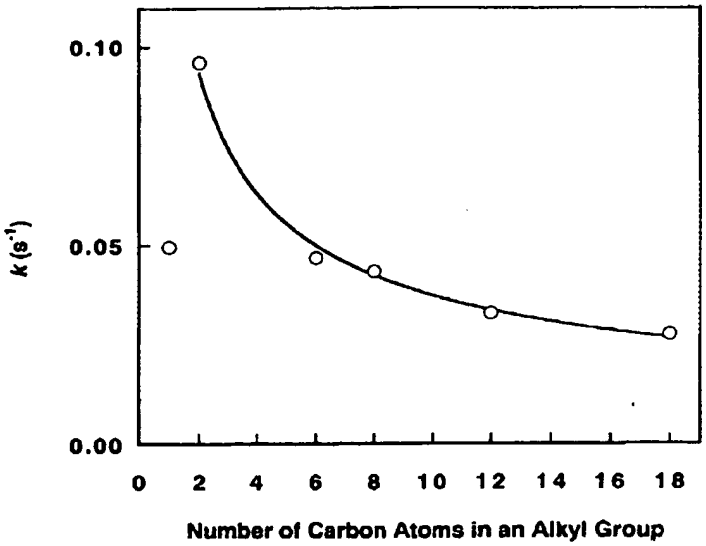


Fig. 2. Effect of chain length on an alkyl group on the rate of photolysis of alkyl-Cbl. Irradiation was carried out in an ice-water bath with a 300-W tungsten light bulb from a distance of 20 cm, and changes in absorbance at 351 nm were measured. k, first-order rate constant.

ring and therefore reassociate with Cbl^{II} more frequently than shorter chain alkyl radicals. The unusually slow rate of photolysis of MeCbl is considered to be due to the relative stability of the methyl radical formed because it lacks the hydrogen to be eliminated on an adjacent carbon atom (*14,15*).

Alkyl-Cbl can be readily converted to dicyano-Cbl by photolysis in the presence of KCN. In the dark, however, long chain alkyl-Cbl in 30% ethanol were fairly stable to 0.1 M KCN (rate constants less than $2\times10^{-2}h^{-1}$).

Conversion of long chain acyl-CN-Cbl to CN-Cbl

Acyl-CN-Cbl underwent hydrolysis in neutral or alkaline solution. The hydrolysis product was confirmed to be CN-Cbl by TLC and spectral measurements. This offers additional evidence to show that an acyl group is bound to CN-Cbl through an ester linkage. To clarify the nature of hydrolysis of acyl-CN-Cbl, the conversion of propionyl-CN-Cbl to CN-Cbl in aqueous solution was kinetically analyzed at 37°C. The rate of hydrolysis followed the pseudo-first order kinetics (Fig. 3) with a rate constant increasing with the pH of the solution ($k'=$ 2.5×10^{-4}, 6.2×10^{-4}, 2.2×10^{-3}, 1.1×10^{-2} and $8.0\times10^{-2}h^{-1}$ at pH 6, 7, 8, 9 and 10, respectively).

Effects of incorporation of methyl group(s) on stability of acyl-CN-Cbl

To improve the lability of acyl-CN-Cbl to hydrolysis, the effects of incorporation of methyl group(s) into the α position of an acyl group were studied. The rates

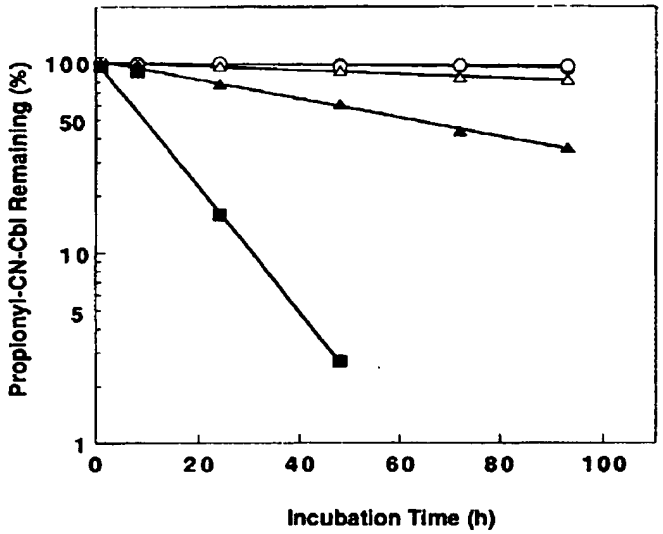


Fig. 3. Effect of pH on the rate of hydrolysis of propionyl-CN-Cbl to CN-Cbl. Propionyl-CN-Cbl was incubated at 37°C for the indicated periods in the following buffers (0.1 M): ○, potassium phosphate buffer (pH 6); ● potassium phosphate buffer (pH 7); △, Tris·HCl buffer (pH 8); ▲, Tris·HCl buffer (pH 9); ■, sodium carbonate buffer (pH 10).

of hydrolysis of pentanoyl-, 2-methylbutyryl- and 2,2-dimethylpropionyl-CN-Cbl at pH 7 and 8 were determined. As described in Fig. 4, 2-methylbutyryl-CN-Cbl was much more stable than pentanoyl-CN-Cbl at both pHs. Therefore, it can be concluded that the introduction of a methyl group into the α position of an acyl group is effective for stabilization toward hydrolysis. However, 2,2-dimethylpropionyl-CN-Cbl was much more labile than the unbranched counterpart, and the time course of hydrolysis was not accurately determined at pH 8. This may be due to steric hindrance induced by the tertiary alkyl group.

Biological activity

Microbiological activities of long chain alkyl-Cbl were determined in the dark with *E. coli* 215, a Cbl- or L-methionine-auxotroph, and *L. leichmannii* as test organisms. As shown in Table 3, all the derivatives tested showed significant growth-supporting activity (Cbl activity). Although the extents of the maximum growth reached with alkyl-Cbl were the same as those obtained with CN-Cbl, there seems to be an optimum length of an alkyl group for the efficiency as Cbl expressed in terms of EC_{50}. Relative microbiological activity of alkyl-Cbl was defined here as $EC_{50,\text{CN-Cbl}}/EC_{50,\text{alkyl-Cbl}}$, which represents the relative efficiency of alkyl-Cbl to that of CN-Cbl. The most active hydrophobic alkyl-Cbl for *E. coli* 215 was octyl- and dodecyl-Cbl which showed ca. 28% activity of CN-Cbl. Toward *L. leichmannii*, longer chain alkyl-Cbl showed higher Cbl activity. Octadecyl-Cbl, the most active

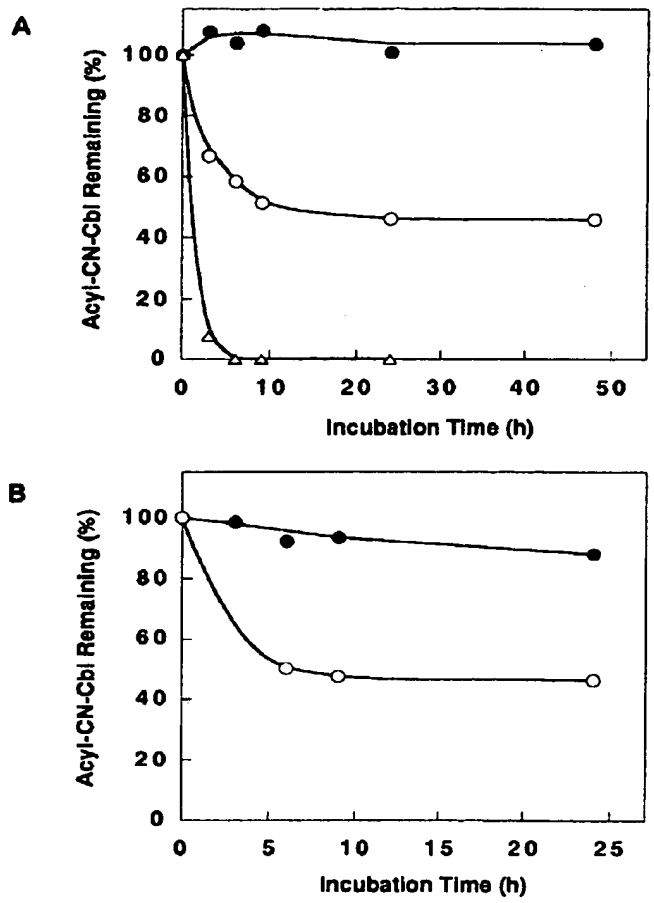


Fig. 4. Effect of incorporation of methyl group(s) into the α position of an acyl group on the rate of hydrolyisis. The experimental conditions are identical to those described in the legend to Fig. 3. A, pH 7; B, pH 8. ○, Pentanoyl-CN-Cbl; ●, 2-methylbutyryl-CN-Cbl; △, 2,2-dimethylpropionyl-CN-Cbl.

Table 3. Microbiological activities of long chain alkyl-Cbl toward *E. coli* 215 and *L. leichmannii*.

Cbl	EC_{50} (nM) (relative efficiency, %)	
	E. coli 215	*L. leichmannii*
CN-Cbl	0.064 (≡100)	0.047 (≡100)
Hexyl-Cbl	0.30 (21)	0.97 (5)
Octyl-Cbl	0.23 (28)	0.91 (5)
Dodecyl-Cbl	0.24 (27)	0.61 (8)
Octadecyl-Cbl	0.55 (12)	0.32 (15)

one, was ca. 15% as active as CN-Cbl.

It is known that Cbl exerts its biological activity by being reduced to Cbl^I and then converted into two coenzyme forms, AdoCbl and MeCbl. The former serves as coenzyme for ribonucleotide reductase of *L. leichmannii* (*17*) and the latter for methionine synthase of *E. coli* (*18*). Therefore, it is likely that a long chain alkyl-Cbl is incorporated into bacterial cells, dealkylated and converted into AdoCbl and MeCbl, although the mechanisms of uptake and dealkylation remain to be elucidated. The optimum length of an alkyl group for the uptake or the dealkylation reaction may be different between *E. coli* and *L. leichmannii*.

Determination of biological activities of long chain acyl-CN-Cbl was difficult, since the derivatives tested were susceptible to hydrolysis even under neutral conditions. Much more stable acyl-CN-Cbl are desirable.

The authors thank A. Iwado for measurement of mass spectra and Y. Kurimoto for her assistance in manuscript preparation.

REFERENCES

1) Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K. (1948): Crystalline vitamin B_{12}. *Science*, **107**, 396–397.

2) Smith, E. L., and Parker, L. F. J. (1948): Purification of anti-pernicious anaemia factor. *Biochem. J.*, **43**, viii.

3) Castle, W. B., Townsend, W. C., and Heath, C. W. (1930): Observations on the etiologic relationship of achylia gastrica to pernicious anemia. *Am. J. Med. Sci.*, **180**, 305–335.

4) Kuovonen, I., and Gräsbeck, R. (1979): A simplified technique to isolate the porcine and human ileal intrinsic factor receptors and studies on their subunit structures. *Biochem. Biophys. Res. Commun.*, **86**, 358–364.

5) Seetharam, B., Alpers, D. H., and Allen, R. H. (1981): Isolation and characterization of the ileal receptor for intrinsic factor-cobalamin. *J. Biol. Chem.*, **256**, 3785–3790.

6) Seligman, P. A., and Allen, R. H. (1978): Characterization of the receptor for transcobalamin II isolated from human placenta. *J. Biol. Chem.*, **253**, 1766–1772.

7) McIntyre, O. R., Sullivan, L. W., Jeffries, G. H., and Silver, R. H. (1965): Pernicious anemia in childhood. *New Engl. J. Med.*, **272**, 981–986.

8) Barker, H. A., Smyth, R. D., Weissbach, H., Munch-Petersen, A., Toohey, J. I., Ladd, J. N., Volcani, B. E., and Wilson, M. R. (1960): Assay, purification, and properties of the adenylcobamide coenzyme. *J. Biol. Chem.*, **235**, 181–190.

9) Ikeda, H. (1956): The microbiological assay of vitamin B_{12} utilizing auxotrophic mutants of *Escherichia coli* (I). Basic study. *Vitamins*, **10**, 268–279.

10) Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K. (1948): The use of *Lactobacillus leichmannii* in the microbiological assay of the "animal protein factor." *J. Biol. Chem.*, **176**, 1459–1460.

11) Ishida, A., Kanefusa, H., Fujita, H., and Toraya, T. (1994): Microbiological activities of nucleotide loop-modified analogues of vitamin B_{12}. *Arch. Microbiol.*, **161**, 293–299.

12) Toraya, T., Ohashi, K., Ueno, H., and Fukui, S. (1975): Preparation, properties and

biological activities of succinyl derivatives of vitamin B_{12}. *Bioinorg. Chem.*, **4**, 245–255.

13) Oenbrink, G., Jürgenlimke, P., and Gabel, D. (1988): Accumulation of porphyrins in cells: influence of hydrophobicity aggregation and protein binding. *Photochem. Photobiol.*, **48**, 451–456.

14) Dolphin, D. H., Johnson, A. W., and Rodrigo, R. (1964): Reactions of the alkylcobalamins. *J. Chem. Soc.*, 3186–3193.

15) Pratt, J. M. (1964): The chemistry of vitamin B_{12}. Part II. Photochemical reactions. *J. Chem. Soc.*, 5154–5160.

16) Hogenkamp, H. P. C., Barker, H. A., and Mason, H. S. (1963): Electron-spin resonance study of coenzyme B_{12}. *Arch. Biochem. Biophys.*, **100**, 353–359.

17) Stubbe, J. (1990): Ribonucleotide reductases. *Adv. Enzymol.*, **63**, 349–419.

18) Banerjee, R. V., and Matthews, R. G. (1990): Cobalamin-dependent methionine synthase. *FASEB J.*, **4**, 1450–1459.

INTER LIBRARY LOAN REQUEST FORM

1105/30

Borrower's Name: Patricia Duffy | Org or A.U.: 1645 | Phone: 305-7555

Serial Number: 09/654,114 | Req. Date: A3AD | Date:

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

71010641 CA: 71(3)10641q JOURNAL
Inhibition of vitamin B12 by an antagonist, cobalamin monocarboxylic acid
AUTHOR(S): Simon, Agoston
LOCATION: Zavod. Farm. Khim. Prod., Budapest, Hung.
JOURNAL: Mikrobiologiya DATE: 1969 VOLUME: 38 NUMBER: 2 PAGES: 211-15
CODEN: MIKBA5 LANGUAGE: Russian
SECTION:
CA808000 Microbial Biochemistry
IDENTIFIERS: inhibitors vitamin B12, antagonists vitamin B12, cobalamin monocarboxylic acid
DESCRIPTORS:
Cobinic acid-pentamide,cyanide hydroxide, dihydrogen phosphate (ester), inner salt, 3'-ester with
5,6-dimethyl-1-.alpha.-D-ribofuranosylbenzimidazole...
vitamin B12 formation inhibition by
CAS REGISTRY NUMBERS:
68-19-9 biological studies, formation of, cobalamin monocarboxylic acid
inhibition of

STIC Use Only 4899391 Accession Number: 397775

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

ндукторов, и определяется соот-
ися в данный момент количест-
уального белка (белков).

Получено 31.VII.1968 г.

'A

: С., Майкелсон М., Соффер Р
нуклеиновых кислот. «Мир», М.
аева И. Н. 1968. Докл. АН СССР,

алимский Н. Д. 1967. Докл. АН

'. J. Mol. Biol., 24, 231.
et biophys. acta, 76, 275.
ol. Biol., 8, 210.
L. I. 1964. J. Mol. Biol., 9, 213.
G., Spahr P. F. a. Watson Y. D.
, 26, 111.
biophys. acta, 55, 139.
a, 49, 64.
Mol. Biol., 6, 341.

L. a. Randall R. I. 1951. J. Biol.

5, 880.
l. Biol., 18, 308.
c M. E. 1965. J. Mol. Biol., 12, 119.
l. Chem., 161, 83.

**SYNTHESIS BY RIBOSOMES
COLI**

G. Orlov

osomes was studied in E. coli M and
s by ribosomes was more or less con-
ges and under various conditions of
ied between the number of ribosomes
oning *m*RNA molecules: *ca.* 10 riboso-
esses of chromosome reduplication and
mRNA synthesis.

Т. XXXVIII *1969* *Вып. 2*

УДК 576.8 : 577.164.16.025

ИНДЕКС ПОДАВЛЕНИЯ ВИТАМИНА B_{12} АНТАГОНИСТОМ — КОБАЛАМИН-МОНОКАРБОКСИЛОВОЙ КИСЛОТОЙ

А. Шимон

Антагонизм витамина B_{12} и кобаламин-монокарбоксиловой кислоты (КМК) имеет своеобразный характер, так как индекс подавления в значительной степени зависит от времени добавления витамина B_{12} и его антагониста к тест-микробу E. coli 113-3. Вероятно, этим особенным характером антагонизма объясняются ошибки при определении индекса подавления и различные значения этого индекса у разных авторов. В настоящем исследовании показано, что при одновременном добавлении витамина B_{12} и КМК индекс 100%-ного подавления равен 60, а индекс 50%-ного подавления равен 35.

Используя в качестве тест-организма штамм E. coli 113—3 удалось показать, что активный изомер кобаламин-монокарбоксиловой кислоты (КМК) является конкурентным ингибитором витамина B_{12} (Smith, 1960). Минимальное отношение концентраций КМК и витамина B_{12}, при котором наблюдается подавление роста на 100 или 50%, называют индексом подавления. При определении индекса подавления разные авторы получали несовпадающие результаты. Так, Смит (Smith, 1962) установил величину индекса, равной 60—100, а Келемен и Шимон (Kelemen a. Simon, 1961) получили для этого же индекса значения 40—60. В то же время Форд (Ford, 1959) нашел, что индекс 50%-ного подавления равен 330, а Гейнрих и Габбе (Heinrich a. Gabbe, 1962) определили индекс равным 110. Эти данные противоречат друг другу, так как индекс 50%-ного подавления должен быть меньше, чем индекс 100%-ного подавления. Разрешению этого вопроса посвящено настоящее исследование.

Материалы и методы исследования

Витамин B_{12}. Препарат фирмы ХИНОИН перекристаллизовывали и чистоту вещества проверяли хроматографией и электрофорезом на бумаге. Концентрацию вещества в растворах определяли спектрофотометрическим методом.

КМК. Использовали продукт фирмы ХИНОИН, изготовленный согласно венгерскому патенту 152.001, для экспериментальных целей. Чистоту КМК проверяли методом хроматографии и электрофореза на бумаге. Анализ показал, что полученное вещество свободно от примесей и является активным изомером антагониста витамина B_{12}, структура которого была установлена Муром с сотрудниками (Moore *et al.*, 1967).

Концентрацию витамина B_{12} определяли методом диффузии в агар, используя E. coli 113-3 в качестве тест-объекта (Чайковская и Дружинина, 1957).

Культивирование штамма E. coli 113-3 проводили на среде следующего состава: метанол — ацетоновый экстракт кукурузы — 3,0 *мл*, KH_2PO_4 — 0,2 *г*; $FeSO_4 \cdot 7H_2O$ — 0,005 *г*, $MgSO_4 \cdot 7H_2O$ — 0,2 *г*, глицерин 2,0 *г*, агар-агар — 2,0 *г*, витамин B_{12} — 2,0 *мкг*, водопроводная вода до 100 *мл*, pH 7,2. Стерилизация среды — 20 мин. при 120°. Бактерии выращивали в пробирках на скошенном агаре при 26° в течение 18—24 час. После инкубации косяки сохраняли 4—6 месяцев в холодильнике при 2—4°.

Для приготовления метанол-ацетонового экстракта кукурузы смешивали 1 *кг* водного кукурузного экстракта (50% сухого вещества) и 1000 *мл* метанола. После стояния в течение 1 часа смесь фильтровали и к фильтрату

добавляли равный объем ацетона. При добавлении ацетона выпадает осадок, который удаляют фильтрованием. Полученный после фильтрования светло-желтый раствор может длительное время без изменения храниться в холодильнике.

Бактерии выращивают в пробирках на среде с 0,5% пептона и 0,5% NaCl в течение 24—48 час. при 26°. Среда практически не содержит витамина B_{12}, в ней определяется около 20—25 *мкг/мл* l-метионина. В опытах, проведенных на агаризованных пластиках, для пересева используют 1% жидкой культуры, для пересева на жидкие питательные среды — 0,2 *мл* той же культуры на 200—300 *мл* среды.

Турбидиметрические и нефелометрические определения проводили на синтетических средах, состав которых приведен у Чайковской и Дружининой (1957) без агара. Для турбидиметрических определений пользовались спектрофотометром типа UNICAM, определение вели при 600 *нм*, а для нефелометрических измерений фотометром типа PULFRICH, снабженным посадкой для нефелометрии. В этом случае используются культуральные жидкости, разведенные в пять раз. За максимальную мутность бактериальных суспензий принимали 100, минимальная (без добавления к среде B_{12}) равнялась 0. Значения, полученные при определении мутностей, выражены в процентах.

Методы определения индекса подавления

а) По Келемен и Шимон (Kelcmen a. Simon, 1961).

В агаризованных пластинках (Чайковская и Дружинина, 1957) вырезаются отверстия, в которые помещаются смеси витамина B_{12} и КМК в различных соотношениях. Отсутствие зон роста показывает 100%-ное подавление.

б) по Смиту (Smith, 1962). На агаризованных пластинках, приготовленных по методу Чайковской и Дружининой (1957), делаются два отверстия, расположенные недалеко друг от друга. В одно отверстие помещается раствор витамина B_{12}, в другое — раствор антагониста.

В этом случае вокруг отверстий агара, содержащих раствор витамина B_{12}, не образуется, как обычно, круговая зона роста E. coli 113-3, а возникает деформированная зона. При этом кажется, что часть зон как бы срезана в том месте, где действует ингибитор. За индекс 100%-ного подавления принимается такое соотношение концентраций B_{12}/КМК, при котором линия подавления находится точно в середине между обоими отверстиями в агаре.

в) Агаризованные пластинки готовятся следующим образом: растворы витамина B_{12} и КМК добавляются к культуре E. coli 113-3 и потом смешиваются с расплавленной агаризованной средой. Витамин B_{12} и КМК могут быть добавлены к культуре бактерий одновременно или последовательно. За 100% подавления принимается полное отсутствие роста бактерий.

г) Производят посев E. coli 113-3 в жидкую питательную среду и добавляют одновременно или последовательно витамин B_{12} и КМК в разных концентрациях. О росте микробной культуры судят по изменению мутности среды, используя для ее определения турбидиметрический или нефелометрический методы.

Результаты

Определение индекса подавления на агаризованной среде

Используя метод, описанный в пункте а, получили значения индекса, равные 50—60, т. е. те же, что и в предыдущих опытах (Kelemen a. Simon, 1961). Полученные данные служили контролем активности препарата КМК, который применялся для последующих опытов. Метод, описанный в пункте б, дал для индекса 100%-ного подавления значения 60. Это значение индекса хорошо совпадает с данными предыдущего опыта и результатами, полученными Смитом (Smith, 1960, 1962).

Метод, описанный в пункте в, дал различные результаты в зависимости от времени добавления витамина B_{12} и КМК к бактериям. Результаты опытов приведены в табл. 1.

Данные табл. 1 показывают, что если витамин B_{12} и КМК добавляются одновременно, то используя метод в, можно получить для индекса 100%-ного подавления те же значения 40—60.

Из табл. 1 следует также, что антагонизм витамина B_{12} и КМК сильно зависит от времени добавления этих веществ в среду. Если КМК добавляли первым, то полное подавление роста получается при 20-кратной концентрации КМК. Если первым добавляется витамин B_{12}, то даже 120-кратная концентрация КМК не вызывает подавления роста. Разница

и ацетона выпадает осадок, который
трования светло-желтый раствор мо-
олодильнике.
с 0,5% пептона и 0,5% NaCl в тече-
ержит витамина B_{12}, в ней определя-
проведенных на агаризованных пла-
ьтуры, для пересева на жидкие пи-
-300 *мл* среды.
деления проводили на синтетических
Дружининой (1957) без агара. Для
спектрофотометром типа UNICAM,
ических измерений фотометром типа
трии. В этом случае используются
. За максимальную мутность бакте-
(без добавления к среде B_{12}) рав-
мутностей, выражены в процентах.

кса подавления

оп. 1961).
Дружинина, 1957) вырезаются отвер-
и КМК в различных соотношениях.
ше.
ных пластинках, приготовленных по
ются два отверстия, расположенные
ается раствор витамина B_{12}, в дру-

щих раствор витамина B_{12}, не обра-
13-3, а возникает деформированная
на в том месте, где действует ингиби-
ется такое соотношение концентра-
ходится точно в середине между

ятся следующим образом:
ьтуре E. coli 113-3 и потом смеши-
итамин B_{12} и КМК могут быть до-
следовательно. За 100% подавления

идкую питательную среду и добав-
B_{12} и КМК в разных концентрациях.
мутности среды, используя для ее
ский методы.

подавления
среде

, получили значения индекса,
их опытах (Kelemen a. Simon,
ролем активности препарата
их опытов. Метод, описанный
явления значения 60. Это зна-
ии предыдущего опыта и ре-
1960, 1962).
ичные результаты в зависи-
и КМК к бактериям. Резуль-

гамин B_{12} и КМК добавляют-
жно получить для индекса
-60.
м витамина B_{12} и КМК силь-
ществ в среду. Если КМК до-
а получается при 20-кратной
ется витамин B_{12}, то даже
т подавления роста. Разница

в 5 мин. при добавлении витамина и его антагониста оказывает влияние на величину индекса подавления. Оказалось, что значение индекса подавления 50—60, полученное методом а или в не абсолютно, и верно только в том случае, когда добавление витамина B_{12} и КМК производится строго одновременно (это достигается тем, что приготовленная смесь обоих соединений добавляется к культуре бактерий).

Таблица 1

Подавляющее действие КМК на активность витамина B_{12} на агаризованной пластинке в случае одновременного и разновременного добавления этих соединений

Отношение концентраций КМК/B_{12}	Время добавления КМК по сравнению с добавлением витамина	Рост культуры E. coli 113—3
10	Одновременно	+
20	»	+
40	»	±
60	»	—
80	»	—
20	5, 10, 20, 40 и 80 мин. раньше	—
120	5,10,20,40 и 80 мин. позже	+

Добавляли каждый раз 1 *мкг/мл* витамина B_{12}. Объяснение знаков +интенсивное развитие бактерий; ± слабое развитие бактерий; — отсутствие развития бактерий.

Влияние КМК на рост E. coli на жидких средах

Подавление роста бактерий в жидких средах изучалось по методу I. В табл. 2 приведены данные, полученные при одновременном добавлении к среде витамина B_{12} и КМК.

Кривые, построенные по данным табл. 2 (рис. 1), почти совпадают. Это означает, что подавляющее действие КМК не зависит от абсолютной концентрации КМК, а только от отношения концентрации КМК и витамин B_{12}. Эти данные подтверждают описанный ранее (Smith, 1960, 1962) конкурентный характер антагонизма этих веществ. Обе кривые пересекают горизонтальную линию, соответствующую 50%-ному росту в той же точке, которая соответствует индексу 50%-ного подавления со значением 35. Этот результат сильно отличается от приведенных ранее данных (110 и 330) и соответствует полученным результатам для индекса 100%-ного подавления, так как индекс 50%-ного подавления меньше 100%-ного подавления. Этот результат следовало ожидать.

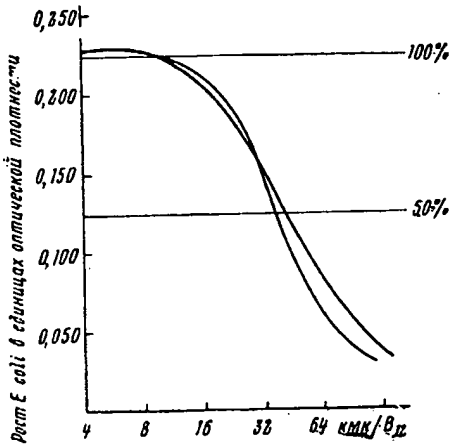


Рис. 1. Подавление роста E. coli 113-3 при различных концентрациях КМК и B_{12} в среде

В другой серии опытов индекс 50%-ного подавления определяли в жидкой среде, витамин B_{12} и КМК добавлялись к культуре не одновременно. Полученные результаты представлены на рис. 2. Кривая *1* соответствует случаю, когда КМК добавляли к культуре E. coli 113—3 на 10 мин. раньше, чем витамин B_{12}. Кривая *3* соответствует результатам опыта, при котором витамин B_{12} добавляли на 10 мин. раньше, чем КМК. Для сравнения приводится кривая *2*, соответствующая одновременному добавлению к культуре КМК и витамина B_{12}.

Если витамин B_{12} добавляется раньше, то 20-кратное количество КМК не вызывает никакого подавления; 40-кратное количество антиви-

Таблица 2

50 %-ное подавление развития культуры E. coli 113—3 кобаламин-монокарбоксиловой кислотой при двух различных концентрациях витамина B_{12} и при одновременном добавлении витамина и антивитамина

Концентрация витамина B_{12}	Отношение концентраций КМК/витамин B_{12}	Поглощение света при 600 *мм*	Концентрация витамина B_{12}	Отношение концентрации КМК/витамин B_{12}	Поглощение света при 600 *мм*
$5 \cdot 10^{-4}$ *мкг/мл*	128	0,090	$5 \cdot 10^{-3}$ *мкг/мл*	128	0,036
	64	0,085		64	0,047
	32	0,143		32	0,146
	16	0,239		16	0,209
	4	0 234		4	0,239
	0	0,233		0	0,224
	50%-ный рост	0,127		50%-ный рост	0,122

Примечание. 50%-ный рост—контрольная культура (100%-ный рост) разбавлена равным количеством стерильной среды. Разбавленная культура содержит половину бактерий по сравнению с исходной культурой.

тамина вызывает слабое подавление роста (12%) и даже 320-кратное количество дает подавление только на 83%. Если КМК добавляется в среду раньше витамина B_{12}, то 2,5-кратное количество вызывает 80%-ное подавление, т. е. такое, которое можно получить при 320-кратном количестве КМК, внесенном, однако, после добавления витамина B_{12}. Даже

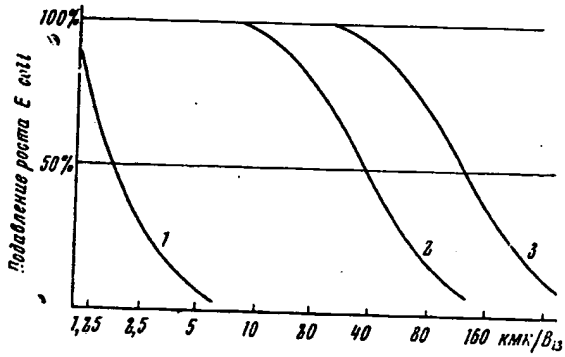


Рис. 2. Подавление роста E. coli 113-3 при разновременном добавлении к культуре бактерий КМК и B_{12}

1— КМК добавляли на 10 мин. раньше; *2*— КМК и B_{12} добавляли одновременно; *3*— витамин B_{12} добавляли на 10 мин. раньше

1,25-кратное количество КМК вызывает 50%-ное подавление, такое, которое получают при 35-кратном количестве КМК, одновременно добавленном с витамином B_{12}.

Приведенные цифры показывают еще нагляднее, чем результаты опытов, проведенных на агаризованных пластинках, что значение индекса подавления сильно зависит от очередности добавления витамина и антивитамина к бактериальной культуре, т. е. от методики определения.

Обсуждение результатов

Антагонизм, существующий между витамином B_{12} и КМК, зависит от порядка добавления обоих веществ к тест-организму. Следовательно, этот антагонизм не является компетитивным в классическом смысле, при котором порядок добавления метаболита и антиметаболита не имеет значения, и активность системы определяется химическим равновесием.

Антагонизм особого типа, наблюдаемый между витамином B_{12} и КМК, не единственный в своем роде. Подобного типа антагонизм наблюдается между β-ацетил-пиридином и никотиновой кислотой (Woolley, 1945, 1952), п-аминофениларзеновой и п-оксибензойной кислотами (Saudgrounq, 1943), 4-аминоптероилглутаминовой и фолиевой кислотами у высших животных (Hertz a. Tullner, 1950) и между фторуксусной

Таблица 2

13—3 кобаламин-монокарбоксиловой гамина B_{12} и при одновременном тивитамина

ция B_{12}	Отношение концентрации КМК/витамин B_{12}	Поглощение света при 600 м.м
	128	0,036
	64	0,047
	32	0,146
	16	0,209
	4	0,239
	0	0,224
	50%-ный рост	0,122

00%-ный рост) разбавлена равным количеловину бактерий по сравнению с исходной

(12%) и даже 320-кратное %. Если КМК добавляется в количество вызывает 80%-ное учить при 320-кратном колиавления витамина B_{12}. Даже

Рис. 2. Подавление роста E. coli 113-3 при разновременном добавлении к культуре бактерий КМК и B_{12}

1— КМК добавляли на 10 мин. раньше; *2*— КМК и B_{12} добавляли одновременно; *3*— витамин B_{12} добавляли на 10 мин. раньше

$/B_{12}$

%-ное подавление, такое, ко-ве КМК, одновременно до-

ляднее, чем результаты опынках, что значение индекса добавления витамина и антиметодики определения.

атов

амином B_{12} и КМК, зависит т-организму. Следовательно, в классическом смысле, при и антиметаболита не имеет ся химическим равновесием. ий между витамином B_{12} и ого типа антагонизм наблюиновой кислотой (Woolley, п-оксибензойной кислотами иновой и фолиевой кислота-950) и между фторуксусной

и уксусной кислотами у дрожжей (Kolnitsky a. Barron, 1947). Несмотря на особенность подобного вида антагонизма, Вулли считает этот тип компентитивным, так как при одинаковых условиях опыта отношение антиметаболита и метаболита определяет активность системы.

Разница в 5 мин. при добавлении метаболита сильно изменяет значение индекса подавления и показывает, что витамин B_{12} и КМК быстро используются E. coli 113—3, хотя эти соединения представляют собой большие молекулы.

Особенным характером антагонизма витамина B_{12} и КМК объясняется тот факт, что разные авторы получали очень различные значения индекса подавления при определении активности этих веществ. Описание опытов, приводимое некоторыми авторами, не позволяет сделать заключение, добавляли ли они витамин B_{12} или его антагонист одновременно. Выше указывалось, что индекс подавления сильно зависит от порядка добавления веществ, это обстоятельство может быть важным источником ошибок при определении. Необходимо обратить внимание на то, что сами тест-микробы, используемые для определения индекса подавления, содержат минимальное количество витамина B_{12}, необходимое для их размножения, что также может влиять на величину индекса подавления. Обычно авторы не обращают внимания на это обстоятельство, хотя оно также должно влиять на точность результатов. Из всего сказанного следует, что стандартизация метода определения индекса подавления антагонистов витамина B_{12} интернациональной организацией является весьма целесообразной.

Завод фармацевтических и химических продуктов
ХИНОИН
Будапешт Венгрия

Получено 20.VI.1968 г.

ЛИТЕРАТУРА

Венгерский патент № 152.001.
Чайковская С. М. и Дружинина Е. Н. 1957. Микробиология, XXVI, 609.
Ford J. E. 1959. J. Gen. Microbiol., 21, 693.
Heinrich H. C. a. Gabbe E. E. 1962. Vitamin B_{12} u. Intrinsic Factor, 2. Europ. Symp.— F. Enke Verlag, Stuttgart. 252.o.
Hertz R. a. Tullner W. W. 1950. Ann. N. Y. Acad. Sci., 52, 1260.
Kalnitsky G. a. Barron N. S. 1947. J. Biol. Chem., 170, 83.
Kelemen A. a. Simon A. Acta Microbiol. 1961. Acad. Sci. Hung., 8, 237.
Moore F. M. Willis B. T. M. a. Hodgkin D. C. 1967. Nature, 214, 129.
Sandground J. H. 1943. J. Pharmacol. and Exptl Therap., 78, 209.
Smith E. L. 1960. Acta Haematol., 24, 9.
Smith E. L. 1962. Vitamin B_{12} u. Intrinsic Factor, 2. Europ Symp.— F. Enke Verlag, Stuttgart, 226.o.
Wooley D. W. 1945. J. Biol. Chem., 157, 455.
Woolley D. W. 1952. A Study of Antimetabolits.

INDEX OF INHIBITION OF VITAMIN B_{12} BY AN ANTAGONIST — COBALAMINE MONOCARBOXYLIC ACID

A. Shimon

1. It was shown that vitamin B_{12} and cobalaminmonocarboxylic acid are competitive antagonists of a special character. The growth — promoting or growth — inhibiting effect of both substances depend not only on the relative concentrations, but also on other factors, namely on which of them is put earlier in contact with the test — organisms.

2. It is supposed that this special character of the antagonism originates the substantial differences, which were found by different authors in the index of inhibition of cobalamin — monocarboxylic acid.

3. When adding vitamin B_{12} and cobalaminmonocarboxylic acid to a culture of E. coli 113—3, the index of 100% inhibition was found to be 60 and the index of 50% inhibition 35.

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Nishizawa etal.
Journal/Book Title:	International journal for vitamin and nutrition research.
Article Title:	
Volume (Issue):	67(3)
Pages:	~~1640~~ 1641-70
Year of Publication:	1997
Publisher:	
Remarks:	

NOS/30
7768256
9202976

STIC Use Only Accession Number: 39783

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date	Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

Internat. J. Vit. Nutr. Res. *67*
(1997) 164–170
Received for publication
December 9, 1996

Methylcobalamin
Vitamin B_{12}
Androgen-sensitive cell line
Estrogen-sensitive cell line

Effects of Methylcobalamin on the Proliferation of Androgen-Sensitive or Estrogen-Sensitive Malignant Cells in Culture and *in vivo*

Yasuko Nishizawa[1], Takashi Yamamoto[1], Nobuyuki Terada[1], Shinji Fushiki[3], Keishi Matsumoto[2] and Yoshio Nishizawa[3]

[1] The Department of Pathology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Nakamichi, Higashinari-ku, Osaka 537, Japan
[2] Osaka Medical Center for Maternal and Child Health, Izumi, Osaka 590-02, Japan
[3] Research Institute for Neurological Disease and Geriatrics, Kyoto Prefectural University of Medicine, Kawaramachi Hirokoji, Kamigyo-ku, Kyoto 620, Japan

Summary: Methylcobalamin is one of the coenzymatically active cobalamin derivates and required for the activity of the cytoplasmic enzyme methionine synthetase catalyzing the methylation of homocysteine into methionine. The effect of methylcobalamin on the proliferation of malignant cells has been examined. Methylcobalamin inhibited the proliferation of androgen-sensitive SC-3 cells (a cloned cell line from Shionogi mouse mammary tumor, SC115) in culture at the concentration of 100–300 µg/ml. An inhibitory activity of methylcobalamin on the proliferation was also observed in other cell lines (estrogen-sensitive B-1F cells from mouse Leydig cell tumor and MCF-7 cells from human mammary tumor) at the concentration of 500 µg/ml. Moreover, large doses of methylcobalamin injected intraperitoneally (100 mg/kg body weight/day) were non-toxic and suppressed the tumor growth of SC115 and B-1F cells in mice fed a vitamin B_{12} deficient diet. These results show that methylcobalamin inhibits the proliferation of malignant cells in culture and in vivo *and propose the possibility of methylcobalamin as a candidate of potentially useful agents for the treatment for some malignant tumors.*

Introduction

Abnormalities of serum levels of vitamin B_{12} and R-binder, one of less specific vitamin B_{12} binding proteins, have been reported in patients with many types of malignant tumor [1, 2]. Serum levels of vitamin B_{12} have been elevated in patients with chronic myeloid leukemia, polycythemia vera and idiopathic thrombocythemia. The presence of abnormal R-binder has been reported in some gastric cancer extracts [3]. Effects of vitamin B_{12} on the proliferation of malignant cells have been also reported. Various reports, however, showed conflicting results. In the treatment of children with neuroblastoma vitamin B_{12} therapy was effective in some studies [4, 5], but not effective in other studies [6, 7]. An inhibitory activity of vitamin B_{12} on the proliferation was shown in experiments with mice and rats. A mixture of vitamins B_{12} and C inhibited the mitotic activity of Ehrlich sarcoma in culture as well as the growth of certain solid tumors [8–10]. In another study with combination of vitamin C, vitamin B_{12} was not effective on the growth of L9 gliosarcoma [11]. In some cases vitamin B_{12} inversely enhanced the growth of fibrosarcoma in rats [12] and exerted a procarcinogenic effect on the induction of malignant tumors in rats and hamsters [13, 14].

A recent study indicated that the metabolically active forms of cobalamins, methylcobalamin

and 5'-deoxyadenosylcobalamin, effectively elongated cell doubling time for the various malignant cells in *in vitro* study [15]. These active metabolites could significantly increase the survival time of animals implanted with the P388 leukemia cells [16]. Furthermore, we have also shown the inhibition of the proliferation of the cell line, established from an androgen-sensitive tumor, by active cobalamins [17]. In addition, cobalamins have inhibited productive human immunodeficiency virus-1 infection of hematopoietic cells *in vitro* [18]. Atypical squamous metaplasia, which is a precancerous lesion, in the lung has disappeared by the administration of the supraphysiological doses of folic acid and vitamin B_{12} to the heavy smokers [19]. In the patients with chronic myelogenous leukemia, the plasma proportion of methylcobalamin was significantly lower than in a reference group, and an association of a low plasma proportion of methylcobalamin with a poor prognosis was shown [20]. In the present study, effects of methylcobalamin on the proliferation of some sex hormone-sensitive cell lines *in vitro* and *in vivo* have been examined to know whether the supraphysiological dose of methylcobalamin is a useful agent for the treatment for some malignant tumors.

Materials and Methods

Cell culture: The cell lines SC-3 and B-1F were established, respectively, from an androgen-sensitive mouse mammary carcinoma SC 115 [21] and from an estrogen-sensitive mouse Leydig cell tumor T124958-R [22]. SC-3 cells were cultured continuously in Eagle's minimum essential medium (MEM) containing 2% dextran-coated charcoal (DCC)-treated fetal bovine serum (FBS) and 10^{-8} M testosterone in the presence of 5% CO_2 in air at 37°C. B-1F cells were maintained in MEM-Ham's F12 (1:1, vol/vol) containing 0.1% (wt/vol) bovine serum albumin (HMB medium) supplemented with 10^{-8} M 17β-estradiol (E_2) in the presence of 5% CO_2 in air at 37°C. Cells were grown to confluence and passaged using trypsin (0.01%, wt/vol)-EDTA (0.02%, wt/vol) in phosphate buffered saline. MCF-7 cells, an estrogen-sensitive human breast cancer cell line, were kindly supplied by Dr. R.L. Sutherland (Garvin Institute of Medical Research, St. Vinsent's Hospital, Sidney, Australia) and maintained in Dulbecco's modified Eagle's medium (DME) containing 10% FBS. MCF-7 cells were passaged using trypsin (0.05%, wt/vol)-EDTA (0.02%, wt/vol) in phosphate buffered saline.

Cell growth experiment: The effects of methylcobalamin on the proliferation were examined as described previously [17, 23–25]. In the present study, the basic medium used for following experiments of B-1F and MCF-7 cells was HMB medium in spite of the presence of cyanocobalamin in HMB medium. Because B-1F and MCF-7 cells could not grow in HMB medium without Ham's F12 and a stimulative effect of estrogen on the proliferation of B-1F cells was not fully observed in the presence of FBS even if at the low concentration. B-1F and MCF-7 cells were plated on four replicate 35-mm culture dishes at an initial cell density of 1×10^4 and 2×10^4/dish in HMB medium and DME containing 5% DCC-treated FBS, respectively. On the following day (day 0), the medium was changed into HMB medium supplemented with various concentrations of methylcobalamin. To remove the residual FBS in full, the dishes on which MCF-7 cells were seeded were washed twice with phosphate-buffered saline. These media were changed every 2 days. On day 6, the viable cells were counted as described previously.

Animals and Diets: Male DS mice (seven-week-old) were obtained from Shionogi Laboratories (Osaka, Japan), housed 5/cage in a temperature-controlled (25°C) room with 12-h light/dark cycle, and given *ad libitum* access to water and pelleted diets. DS mice were transplanted subcutaneously with minced SC115 tumors. Starting 24 hours (day 0) after transplantation, mice were injected intraperitoneally with or without methylcobalamin (100 mg/kg body weight/day) in 0.3 ml of saline solution daily for 24 days. Sterile methylcobalamin solution (10 mg/ml of saline) was used. Feeding with vitamin B_{12} deficient diets was started simultaneously with the injection. Pelleted vitamin B_{12} deficient diets (Clea Japan Inc. Tokyo, Japan) (Tab. I) were stored at 4°C and given with every other day replacement. Palpable tumor was measured with caliper at the indicated times. In case of B-1F cells, after being har-

Table I: Nutrient composition of vitamin B_{12} deficient diet

Vitamin-free casein	18.0%
Sucrose	67.6%
Corn oil	8.0%
Hegsted salt mixture	4.0%
Avisel	1.5%
DL-methionine	0.3%
Choline chloride	0.1%
Vitamin mixture	0.5%
Vitamin A, D_3 (500 000 IU/100 000 IU)	2.00 mg/100 g of diet
Vitamin A (500 000 IU/g)	2.00
Vitamin E (50%)	20.00
Vitamin B_1	1.50
Vitamin B_2	0.80
Vitamin B_6	0.50
Vitamin C-Ca	24.35
Vitamin K_3	0.50
Biotin (2%)	2.00
DL-Ca-pantothenate	8.00
PABA	10.00
Nicotinic acid	5.00
Inositol	10.00
Folic acid	0.20
Lactose	413.15

vested and washed, the cells (5×10^5 cells) resuspended in MEM were implanted subcutaneously into castrated male Balb/c mice (seven-week-old). The Balb/c mice were implanted subcutaneously with a fused pellet of 5% estradiol in cholesterol and injected intraperitoneally with or without methylcobalamin on the following day (day 0) of B-1F cell implantation.

Vitamin B_{12} assay: In principle, vitamin B_{12} assay was performed as described by Gimsing [20]. On day 24 the mice described above were sacrificed under nembtal anesthesia. The whole blood samples were taken via an inferior *vena cava* by venopuncture. The serum was separated after centrifugation within 30 min after sampling. Syringes and sample containers were covered with aluminium foil. All stages of the method were carried out by red safe-light or in darkness. Tumors were cut into small pieces and washed twice with 0.15 M NaCl at 4°C. Minced tissues were homogenized in four volumes of 0.15 M NaCl by Polytron PT-10. After centrifugation (10 min, 2000 g) of homogenates the supernatants were used for vitamin B_{12} and protein assays. The concentration of protein was determined using protein assay kit. The total concentration of vitamin B_{12} was determined by radioisotope dilution method using a solid-phase intrinsic factor as the specific cobalamin-binding protein. The assays were performed as directed in the manual from the supplier of the assay kit. For vitamin B_{12} the intra-assay variation amounted to a mean of 2–6% and the inter-assay variation to a mean of 5–8%. In case of established cells, cells were seeded at an initial density of 1.3 and 2.6×10^6 cells/100-mm dish in MEM containing 2% DCC-treated fFBS and HMB medium, respectively, for SC-3 and B-1F cells. On the following day (day 0), the medium was changed to 2% DCC-treated FBS-MEM supplemented with 10^{-6} M testosterone (SC-3 cells) and 10^{-9} M estradiol (B-1F cells) in the presence or absence lof methylcobalamin (final concentration of 300 μg/ml for SC-3 cells or 500 μg/ml for B-1F cells). The cells harvested on day 3 were washed four times with 20 ml of ice-cold 0.15 M NaCl and then resuspended in four volumes of 0.15 M NaCl. The viable cell number was determined with hemocytometer by trypan blue dye exclusion method. The cells lyzed by freeze-thawing were homogenized as described for minced tissues.

Statistical analysis: All values were expressed as mean ± SE. Significant differences were estimated by Student's t test ($p<0.05$).

Materials: The radioinert steroids, BSA, trypsin, activated charcoal and methylcobalamin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dextran T-70 was from Pharmacia Fine Chemicals (Uppsala, Sweden). MEM, DME and Ham's F-12 were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). FBS was from Nichirei Co., Ltd. (Tokyo, Japan). M-vitamin B_{12}/ folic acid kit was from CHIRON (Tokyo, Japan). Protein assay kit was from BIO-RAD (California, USA). Balb/c mice were obtained from Shizuoka Experimental Animal Farm (Shizuoka, Japan). The other chemicals used in this study were of analytical grade.

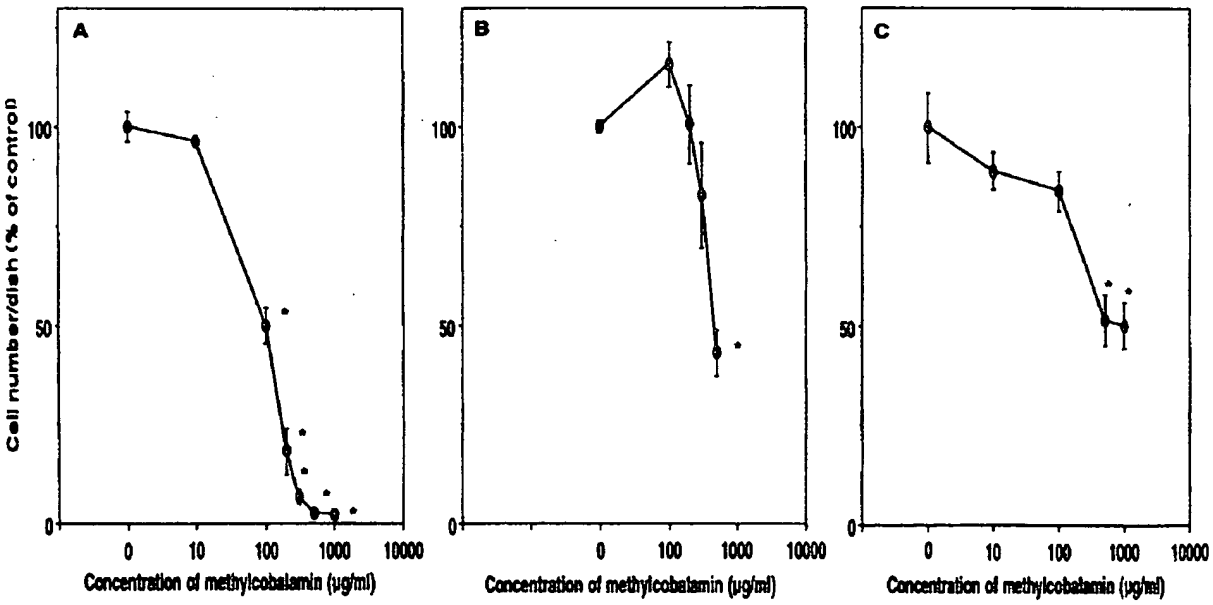


Figure 1: Effects of methylcobalamin on the proliferation of SC-3, B-1F and MCF-7 cells.
Cells were plated on four 35-mm culture dishes as described in materials and methods. On the following day (day 0), the medium was changed into the medium supplemented with various concentrations of methylcobalamin. On day 6 the viable cells were counted. The cell number was expressed as a percentage, taking the value in the absence of methylcobalamin as 100%. A, B and C correspond to SC-3, B-1F and MCF-7 cells respectively. Points, means of 4 determinations; bars, S. E.; *, $P<0.05$. Further 4 trials gave similar results.

Results

Effects of methylcobalamin on the proliferation of cultured cells: Figure 1 presents results on the cell yield of androgen-sensitive SC-3 cells and estrogen-sensitive B-1F and MCF-7 cells treated with various concentrations of methylcobalamin. In SC-3 cells (Fig. 1A) methylcobalamin inhibited the proliferation at the concentrations of 50–100 μg/ml (0.037–0.074 mM) as reported previously [17]. The proliferation of B-1F and MCF-7 cells were also inhibited (Fig. 1B and 1C) by methylcobalamin, but a higher concentration (500 μg/ml, 0.37 mM) of methylcobalamin was necessary.

Effects of methylcobalamin on the tumor growth: Effects of methylcobalamin on the tumor growth were examined at the dose of 100 mg/kg body weight/injection/day. The mice were able to tolerate the injection of methylcobalamin at this dose without any adverse effects. In the present study B-1F cells were used *in vitro* (in culture) and *in vivo*. In the experiments of androgen-sensitive tumor, tumor pieces of SC115 were subcutaneously transplanted instead of SC-3 cells, because SC-3 cells could not cause the tumor formation *in vivo*. Figure 2 shows an inhibitory effect of methylcobalamin on the tumor growth of SC115, especially in early phase, in DS mice. In B-1F cells (Fig. 3) methylcobalamin also inhibited the tumor growth. The ratio of the tumor formation was not significantly different between control and methylcobalamin injected mice (SC115 and B-1F cells). In histological findings with hematoxylin-eosin stain there was no prominent difference between tumors treated with and without methylcobalamin (data not shown), and no toxic effect on mice was observed.

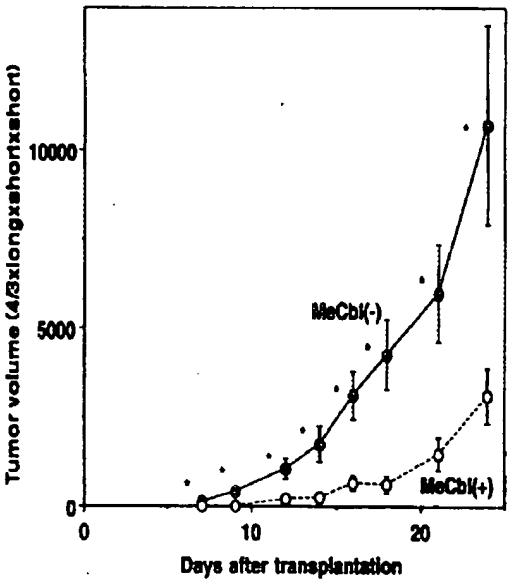


Figure 2: In vivo growth inhibition of SC115 tumor by methylcobalamin.
Male DS mice (seven-week-old) were transplanted subcutaneously with minced SC115 tumors. Starting 24 hours (day 0) after transplantation, mice were injected intraperitoneally with or without methylcobalamin (100 mg/kg body weight/day) daily for 24 days. Feeding with vitamin B_{12} deficient diets was started simultaneously with the injection. Palpable tumor was measured at the indicated times. The tumor volume is expressed as 4/3 × long diameter (mm) × short diameter (mm) × short diameter (mm). Points, means of 10 determinations; bars, S.E.; *, $P<0.05$; MeCbl, methylcobalamin. Further 3 trials gave similar results.

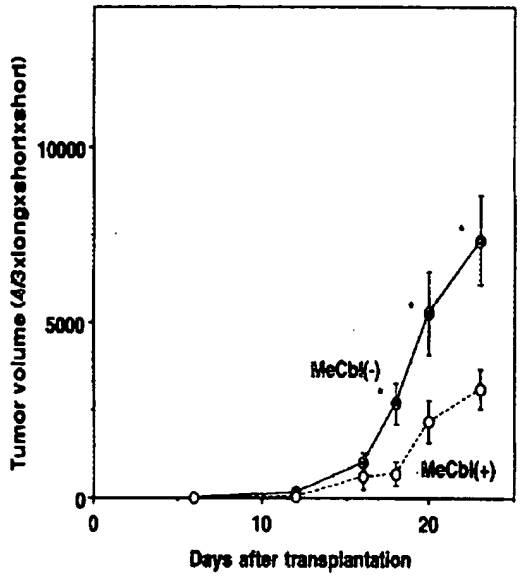


Figure 3: In vivo growth inhibition of B-1F tumor by methylcobalamin.
B-1F cells (5×10^3 cells) in MEM were implanted subcutaneously into castrated male Balb/c mice (seven-week-old). The mice were also implanted subcutaneously with a fused pellet of 5% estradiol in cholesterol and injected intraperitoneally with or without methylcobalamin on the following day (day 0) of B-1F cell implantation. Feeding with vitamin B_{12} deficient diets was started simultaneously with the injection. Palpable tumor was measured at the indicated times. The tumor volume is expressed as 4/3 × long diameter (mm) × short diameter (mm) × short diameter (mm). Points, means of 10 determinations; bars, S.E.; *, $P<0.05$; MeCbl, methylcobalamin. Further 3 trials gave similar results.

Vitamin B_{12} assay: Sera and tissue specimens of the mice, and cultured cells treated with or without methylcobalamin were measured for total vitamin B_{12}, using M-vitamin B_{12}/folic acid kit. The concentrations of vitamin B_{12} are given in Table II. The serum, tissue and cellular concentrations of vitamin B_{12} were significantly increased in cases treated with methylcobalamin compared to the controls.

Discussion

We have found that methylcobalamin inhibits the *in vitro* proliferation of the androgen-sensitive cell line SC-3 and of the estrogen-sensitive cell lines B-1F and MCF-7 as well as the *in vivo* tumor growth of SC115 and B-1F cells. Effective dose of methylcobalamin was higher in B-1F and MCF-7 cells than in SC-3 cells. It might be partly due to the difference of the intracellular vitamin B_{12} contents among these cells. The basal level of vitamin B_{12} in SC-3 cells was extremely lower than that in B-1F cells. In the *in vitro* experiments the basic culture medium used for B-1F and MCF-7 cells was a HMB medium containing a cyanocobalamin at the concentration of 0.68 μg/ml. On the other hand the medium used for SC-3 cells contained vitamin B_{12} at the concentration of 10–30 pg/ml. Methylcobalamin seems to be also more effective for SC115 tumor than for B-1F tumor. The content of vitamin B_{12} in SC115 tumor was lower than that in B-1F tumor, while the serum levels of vitamin B_{12} were similar between Balb/c and DS mice. The metabolism of methylcobalamin might be different between SC115 and B-1F tumors, therefore between SC-3 and B-1F cells. In the *in vivo* experiments the observed maximum inhibitory activity of methylcobalamin was smaller than that in cultured systems, partly due to the solubility of methylcobalamin. It could not be solubilized in concentrations larger than 10 mg/ml in PBS. Therefore it was difficult that mice could be administered with methylcobalamin at doses larger than 100 mg/kg body weight/day in 0.3 ml of saline solution.

The precise mechanism of the inhibitory activity of methylcobalamin is not clear. In SC-3 cells, androgen induces FGF-like growth factor (AIGF) and FGF receptors [26, 27]. In MCF-7 cells, estrogen induces various growth factors

Table II: Concentration of vitamin B_{12} in tissue, serum and cells

	Methylcobalamin (−)	Methylcobalamin (+)
SC115 tumor		
Tumor	0.39±0.04 ng/mg protein (n=8)	13.28±1.34 ng/mg protein (n=6)
Serum	14276±3287 pg/ml (n=5)	204061±12506 pg/ml (n=5)
B-1F tumor		
Tumor	1.56±0.12 ng/mg protein (n=8)	22.76±3.48 ng/mg protein (n=7)
Serum	15035±970 pg/ml (n=10)	183000±16197 pg/ml (n=9)
SC-3 cell		
	0.01±0.00 ng/10^6 cells (n=5)	148.6±12.6 ng/10^6 cells (n=5)
	0.10±0.03 ng/mg protein (n=5)	1871±101 ng/mg protein (n=5)
B-1F cell		
	0.24±0.01 ng/10^6 cells (n=5)	230±13.5 ng/10^6 cells (n=5)
	1.81±0.02 ng/mg protein (n=5)	2246±120 ng/mg protein (n=5)

On day 24 the mice described in Figures 2 and 3 were sacrificed. The whole blood samples were taken via an inferior *vena cava* by venopuncture. In case of established cells, cells were seeded and treated with methylcobalamin as described in materials and methods. The total concentration of vitamin B_{12} in tumors, sera and cells was determined by radio isotope dilution method. Table shows means±S.E. "n" shows the number of assayed samples.

and their receptors [28–30]. But estrogen inhibits the activity of 5-lipoxygenase, which is a key enzyme for the production of leukotrienes that suppress the proliferation of B-1F cells [23, 24, 31, 32]. In SC-3 and MCF-7 cells, and SC115 tumor, sex-hormone seems to mostly stimulate the positive growth control system, while estrogen seems to mostly inactivate the negative growth control system in B-1F cells. The inhibitory activity of methylcobalamin on the proliferation might be mediated through the interference of these systems. In our previous report [17] the treatment of SC-3 cells with methylcobalamin decreased the activity of AIGF at the level of post androgen receptor binding. The presence of the common inhibitory mechanism by methylcobalamin among these diverse systems is unknown. Furthermore, whether the inhibitory activity is specific for methylcobalamin or not is also unknown up to this point. Nevertheless, because of the ability to achieve high blood and tissue levels *in vivo* and the lack of toxicity, methylcobalamin should be considered as a candidate of potentially useful agents for the treatment for some malignant tumors.

Acknowledgement: The technical assistance of M. Tani, K. Morimoto and H. Ogasawara is gratefully acknowledged. This study was supported by grants-in-aids from the Research Foundation for Cancer and Cardiovascular Diseases, Osaka, Japan.

References

1. Beard, B., Pitney, W.R. and Sanneman, E.H. (1954) Serum concentrations of vitamin B_{12} in patients suffering from leukemia. Blood *9*, 789–794.
2. Nelson, R. and Doctor, V.M. (1962) Vitamin B_{12} content of liver and serum in malignant neoplasia. Gastroenterology *42*, 414–418.
3. Wakatsuki, Y., Inada, M., Kudo, H., Shio, G., Masuda, T., Miyake, T. and Kita, T. (1989) Immunological characterization and clinical implication of cobalamin binding protein in human gastric cancer. Cancer Res. *49*, 3122–3128.
4. Bodian, M. (1959) Neuroblastoma. Pediatr. Clin. N. Amer. *6*, 449–472.
5. Bodain, M. (1963) Neuroblastoma, an evaluation of its natural history and the effects of therapy, with particular reference to treatment by massive doses of vitamin B_{12}. Arch. Dis. Childh. *38*, 609–619.
6. Sawitcky, A. and Desposito, F. (1965) A survey of American experience with vitamin B_{12} therapy of neuroblastoma. J. Pediatr. *67*, 99–103.
7. Landerman, M.J.S. (1970) Treatment of neuroblastoma with vitamin B_{12}. Arch. Dis. Childh. *45*, 385–387.
8. Poydock, M.E., Fardon, J.C., Gallina, D., Ferro, V. and Heher, C. (1979) Inhibiting effect of vitamins C and B_{12} on the mitotic activity of ascites tumors. Exp. Cell. Biol. *47*, 210–217.
9. Poydock, M.E., Phillips, L. and Schmitt, P. (1984) Growth-inhibiting effect of hydroxocobalamin and L-ascorbic acid on two solid tumors in mice. I.R.C.S. Med. *12*, 813.
10. Poydock, M.E., Harguindey, S., Hart, T., Takita, H. and Kelly, D. (1985) Mitogenic inhibition and effect on survival of mice bearing L1210 leukemia using a combination of dehydroascorbic acid and hydroxycobalamin. Am. J. Clin. Oncol. (CCT) *8*, 266–269.
11. Newell Jr., S.D., Kapp, J. and Romfh, J.H. (1981) Evaluation of megadose vitamin therapy in an experimental brain tumor. Surg. Neurol. *16*, 161–164.
12. Rigby, C.C. and Bodian, M. (1963) Experimental study of the relationship between vitamin B_{12} and two animal tumour systems. Br. J. Cancer *17*, 90–99.
13. Day, P.L., Payne, L.D. and Dinning, J.S. (1950) Procarcinogenic effect of vitamin B_{12} on p-dimethylaminoazobenzene-fed rats. Pro. Soc. Exp. Biol. N. Y. *74*, 854–855.
14. Georgadze, G.E. (1960) The influence of vitamins B_1 and B_{12} on the induction of malignant tumours in hamsters. Vopr. Onkol. *6*, 54–58.
15. Tsao, C.S., Miyashita, K. and Young, M. (1990) Cytotoxic activity of cobalamin in cultured malignant and nonmalignant cells. Pathobiology *58*, 292–296.
16. Tsao, C.S. and Miyashita, K. (1993) Influence of cobalamin on the survival of mice bearing ascites tumor. Pathobiology *61*, 104–108.
17. Nishizawa, Y., Yamamoto, T., Terada, N., Sato, B., Matsumoto, K. and Nishizawa, Y. (1995) Inhibition of the proliferation of androgen-dependent SC-3 cells by methylcobalamin. Oncology Reports *2*, 129–132.
18. Weinberg, J.B., Sauls, D.L., Misukonis, M.A. and Shugars, D.C. (1995) Inhibition of productive human immunodeficiency virus-1 infection by cobalamins. Blood *86*, 1281–1287.
19. Heimburger, D.C., Alexander, C.B., Birch, R., Butterworth Jr., C.E., Bailley, W.C. and Krumdieck, C.L. (1988) Improvement in bronchial squamous metaplasia in smokers treated with folate and vitamin B_{12}. JAMA *259*, 1525–1530.
20. Gimsing, P. (1995) Cobalamin forms and analogues in plasma and myeloid cells during chronic myelogenous leukemia related to clinical condition. Br. J. Haematology *89*, 812–819.
21. Noguchi, S., Nishizawa, Y., Nakamura, N., Uchida, N., Yamaguchi, K., Sato, B., Kitamura, Y. and Matsumoto, K. (1987) Growth-stimulating effect of pharmacological doses of estrogen on androgen-dependent Shionogi carcinoma 115 *in vivo* but not in cell culture. Cancer Res. *47*, 263–268.
22. Nishizawa, Y., Sato, B., Miyashita, Y., Tsukada, S., Hirose, T., Kishimoto, S. and Matsumoto, K. (1988) Autocrine regulation of cell proliferation by estradiol and hydroxytamoxifen of transformed mouse Leyding cells in serum-free culture. Endocrinology *122*, 227–235.
23. Nishizawa, Y., Nishii, K., Nishizawa, Y., Koga, M., Kishimoto, S., Matsumoto, M. and Sato, B. (1990) Effects of estrogen on cell proliferation and leukotriene formation in transformed mouse Leydig cells cultured under serum-free conditions. Cancer Res. *50*, 3866–3871.
24. Nishii, K., Nishizawa, Y., Nishizawa, Y., Matsumoto, K. and Sato, B. (1991) Inhibition of murine transformed Leydig cell proliferation by leukotrienes in serum-free culture. Cancer Res. *51*, 5573–5578.
25. Nishizawa, Y., Yamamoto, T., Terada, N., Fushiki, S., Amakata, Y. and Nishizawa Y. (1996) Effects of antialler-

gic drugs on the proliferation of estrogen-sensitive mouse Leydig cell line. Anticancer Res. *16*, 1241–1246.
26. Tanaka, A., Miyamoto, K., Minamino, N., Takeda, M., Sato, B., Matsuo, H. and Matsumoto M. (1992) Cloing and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells. Proc. Natl. Acad. Sci. USA *89*, 8928–8932.
27. Kouhara, H., Kasayama, S., Saito, H., Matsumoto, K. and Sato, S. (1991) Expression cDNA cloning of fibroblast growth factor (FGF) receptor in mouse breast cancer cells: A variant form in FGF-responsive transformed cells. Biochem. Biophys. Res. Commun. *176*, 31–37.
28. Vignon, F., Derocq, D. F., Chambon, M. and Rochefort, H. (1983) Estrogen induced proteins secreted by MCF-7 human breast cancer cells. C.R. Acad. Sci. Paris Endocrinol. *296*, 151–157.
29. Vignon, F., Capony, F., Chambon, M., Freiss, G., Marcia, M. and Rochefort, H. (1986) Autocrine growth stimulation of the MCF-7 breast cancer cell by the estrogen-regulated 52K protein. Endocrinology *118*, 1537–1545.
30. Dickson, R.B., Huff, K.K., Spencer, E.M. and Lippman, M.E. (1986) Induction of epidermal growth factor-related polypeptides by 17β-estradiol in MCF-7 human breast cancer cells. Endocrinology *118*, 138–142.
31. Nishizawa, Y., Nishii, K., Kihsimoto, S., Matsumoto, K. and Sato, B. (1990) Regulatory role of arachidonic acid-derived matabolites for proliferation of transformed murine Leydig cell in serum-free culture condition. Anticancer Res. *10*, 317–322.
32. Nishizawa, Y., Yamamoto, T., Taniguchi, H. and Sato, B. (1992) Effects of estrogen and leukotrienes on anchorage-independent growth of transformed mouse Leydig cells under serum-free condition. Steroid Biochem. (Life Sci. Adv.) *11*, 23–28.

Dr. Yasuko Nishizawa, The Department of Pathology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Nakamichi, 1-3-3, Higashinari-ku, Osaka, 537, Japan

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name: Patricia Duffy | Org or A.U.: 1645 | Phone: 305-7555

Serial Number: 09/654,114 | Req. Date: ASAP | Date:

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	McLean et al.
Journal/Book Title:	Leukemia + Lymphoma
Article Title:	
Volume (Issue):	30 (1-2).
Pages:	101 – 109.
Year of Publication:	1998
Publisher:	
Remarks:	

N05/30

7768029

969680

STIC Use Only Accession Number: 29778S

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

Leukemia and Lymphoma, Vol. 30, pp. 101–109
Reprints available directly from the publisher
Photocopying permitted by license only

Transcobalamin II and *in vitro* Proliferation of Leukemic Cells

G. R. McLEAN[a], M. J. WILLIAMS[a], C. S. WOODHOUSE[b,c] and H. J. ZILTENER[a,d,*]

[a]*The Biomedical Research Centre, University of British Columbia, Vancouver, BC,* [b]*Receptagen Corp., Edmonds, WA,* [c]*Department of Radiation Oncology, University of Washington, Seattle, WA, USA and* [d]*Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver BC, Canada*

(Received 31 August 1997)

We have recently shown that antibodies to transcobalamin II (TCII) inhibit the *in vitro* growth of human and murine leukemic cells. This antiproliferative strategy targets the uptake of cobalamin (Cbl), an essential cofactor for two biochemical reactions in humans. To date there has been no appropriate cell culture model available to study antagonism of Cbl as a potential antiproliferative strategy. We have established cell culture conditions which allow reproducible measurements of cell proliferation that is dependent on Cbl and its carrier protein, TCII. This bioassay has allowed us to demonstrate that several monoclonal antibodies, raised against TCII, are potent inhibitors of cell proliferation and that excess Cbl abrogates this inhibitory effect. Thus, supporting our hypothesis that interference with Cbl uptake or metabolism will result in inhibition of cell proliferation. Furthermore, Cbl metabolism appears to provide a useful target for antiproliferative strategies which now involve the use of inactive Cbl analogs. In this review, we update our work on the role of targeting TCII and Cbl as an antiproliferative strategy for leukemic cells. We suggest that this strategy may provide a novel direction for anti cancer reagents.

Keywords: Transcobalamin II, proliferation of leukemic cells

INTRODUCTION

Cobalamin (Cbl), also known as vitamin B12, is derived from the diet and binds to transcobalamin II (TCII), one of at least three Cbl binding proteins, which facilitates its uptake by cells and peripheral tissues.[1] TCII, a non glycosylated plasma protein of 43kDa,[2] binds specific cell surface receptors that recognise holoTCII (complex of Cbl-TCII) in preference to apoTCII (free TCII).[3] Internalisation of Cbl-TCII is achieved by endocytosis of the receptor complex. Recently Bose et al[4] showed that the human

*Corresponding author. Fax: 604 822-7815. E-mail: hermann@brc.ubc.ca.

placenta derived TCII receptor exists as a monomer of 62kDa in microsomal membranes and as a dimer of 124kDa in plasma membranes. Furthermore, the dimerisation of the TCII receptor was dependent on membrane rigidity which is itself determined by cholesterol levels.

Intracellular Cbl metabolism involves conversion of the vitamin to coenzyme forms via a number of enzymatic reactions.[5] Internalised Cbl is released from TCII within the lysosome, transported to the cytoplasm where it is reduced by specific reductases from the +3 oxidation state to the +2 or +1 state, and then further modified to the coenzyme forms. In humans these coenzyme forms of Cbl bind to and activate two enzymes, methionine synthase (MS) which is localised in the cytoplasm, and methylmalonyl-CoA mutase (MCM) which is localised in mitochondria. MS is the most promising target affected by Cbl deficiency since this enzyme is linked to folate metabolism and to thymidine and DNA synthesis.[6] Inhibition of MS by blocking Cbl uptake or supplying inactive Cbl analogs may thus result in impaired DNA synthesis and provide a means to block cell proliferation.

Indeed, there are many examples indicating that impaired Cbl metabolism results in reduced cell proliferation and may be beneficial in cancer treatment. Prolonged nitrous oxide (N_20) treatment was recognised many years ago to result in myelosuppression[7] and there are many studies demonstrating the cytotoxic effects of N_2O treatment.[8–10] The inhibitory effect of nitrous oxide is due to the irreversible oxidation of cobalt I to cobalt III in Cbl and the consequent loss of MS activity.[11] This deficiency in functional Cbl induced by N_2O, results in blocked DNA synthesis via inhibition of MS activity and is thought to be the basis of the effect of N_2O in treatment of cancer.[12] N_2O treatment of patients was used as early as 1959 in initial attempts to treat chronic myelogenous leukaemia (CML)[13] and later to treat patients with acute myelogenous leukaemia (AML).[14] Effects of N_2O treatment have also been explored in rodent models.[15–18] Combination of N_2O treatment with other antiproliferative drugs which interfere with folate metabolism (such as methotrexate and 5-fluorouracil), increased the anti-leukemic effect.[19–20] Combinations of cytotoxic drugs with N_2O treatment are also particularly promising for the successful treatment of cancer in humans, as shown recently in a case where a synergistic effect of methotrexate and N_2O treatment was found.[21] However, the use of N_2O as an antiproliferative agent is not ideal even though these results indicate that inhibition of Cbl metabolism may offer a novel approach to inhibit leukemic cell growth. We now have used the approach of inhibiting Cbl uptake using anti-TCII monoclonal antibodies and have been able to demonstrate, consistent with the observations described above, that inhibition of Cbl uptake results in inhibition of cell proliferation. Moreover, it was recently shown that depletion of Cbl induces apoptosis in neoplastic cells.[22]

RESULTS AND DISCUSSION

In order to demonstrate the *in vitro* growth dependence of human and murine leukemic cells to Cbl and/or TCII, we have developed a bioassay system using a specially designed growth medium.[25] Using this bioassay we have been able to show that proliferation of leukemic cells can be made dependent on Cbl and/or holoTCII *in vitro*. Furthermore, we examined several monoclonal antibodies raised against human TCII[24] for their ability to block TCII dependent cell growth.[25] Here we describe further work investigating Cbl/TCII dependent leukemic cell proliferation *in vitro*.

MATERIALS AND METHODS

Materials

The mouse lymphoma cell line BW5147 and the mouse mastocytoma cell line P815 were from ATCC (Rockville, MD). The human erythroluekemic cell line K562 was from NRC (Ottawa, Canada). RPMI 1640 culture medium, RPMI 1640 culture medium deficient in cobalamin and folic acid, and RPMI 1640 culture medium deficient in methionine, cobalamin and folic acid were obtained from Stem Cell Technologies

(Vancouver, Canada). Fetal calf serum (FCS) was from GIBCO (Grand Island, NY) and human serum (HuS) from ICN Biomedicals Inc (Costa Mesa, CA). Microfine precipitated silica (QUSO) was a gift from Degussa Corp (Ridgefield, NJ). Cyanocobalamin, 5-methyltetrahydrofolic acid, and DL-homocysteine were obtained from Sigma Chemical (St Louis, MO). Radiolabelled cyanocobalamin (^{57}Co-Cbl) 10.5uCi/ml was purchased from Amersham (Oakville, Canada). Bacto agar was from DIFCO Laboratories (Detroit, MI). Recombinant human TCII (rhTCII) was produced as described previously.[23] The anti-human TCII monoclonal antibodies have been described and characterised previously.[20,25]

Cell Culture

W5147, P815 and K562 cells were maintained in complete RPMI 1640 medium supplemented with 10% FCS in 60 × 15 mm culture dishes in a humidified atmosphere (5% CO_2, 95% Air) at 37°C. Cells used in the Cbl/TCII bioassay were grown to late log phase then washed three times in phosphate-buffered saline (PBS) before resuspension in Cbl-deficient bioassay medium.

Cbl/TCII Bioassay

The bioassay to determine Cbl/TCII dependent cell growth has been described in detail previously.[25] In all cases, cell viability was assessed by MTT reduction as previously described.[26-27]

Colony Forming Assay

Cells were treated as for the Cbl/TCII bioassay except that they were seeded at 1000 cells per 35 × 10 mm dish (Nunc InterMed, Roskilde, Denmark) in medium containing 0.3% (w/v) bacto agar. Colonies were counted after 7 days in culture.

^{57}Co-Cbl-TCII Binding Assay

100ul of human serum was labelled with 2ul of ^{57}Co-Cbl and incubated at 37°C for 1 hour. To this mixture, 10^6 K562 cells in PBS were added and incubated for a further 1 hour before washing 3 times with PBS. Antibodies were included at 10ug/ml. To determine specific binding of labelled TCII to the cells, the resuspended cell pellets were assessed for γ radiation in a γ counter.

In vitro TCII Growth Dependence of Murine and Human Cell Lines

Previously we have shown that the viability of human and murine leukemic cells *in vitro* was dependent on the presence of Cbl and/or TCII in the growth medium.[25] In order to show that murine leukemic cell colony growth *in vitro* was also dependent on Cbl and TCII, we adapted our bioassay conditions from liquid suspension cultures to semisolid agar cultures. Figure 1 shows that the colony growth of murine lymphoma BW5147 cells is dependent on holoTCII *in vitro*. As expected, the colonies that developed in the absence of holoTCII were considerably smaller than the colonies grown in the presence of holoTCII. Generally we observed 3–4 fold decreases in the number of colonies in the absence of holoTCII when colonies were grown in the standard bioassay medium which lacks Cbl, folic acid, bovine TCII (from FCS) and is supplemented with homocysteine and 5-methyltetrahydrofolic acid (Fig 1A). When this bioassay growth medium in addition lacked methionine we found an absolute dependence on TCII for colony formation (Fig 1B). Thus, these results confirm our already reported data that optimal leukemic cell growth *in vitro* requires Cbl and TCII, and further indicates that under extreme conditions (lack of methionine) cell growth absolutely requires Cbl and TCII.

Optimisation of Cbl/TCII Dependent *In Vitro* Growth for Leukemic Cell Lines

Using the established bioassay conditions, we have demonstrated Cbl/TCII dependent *in vitro* growth of the murine mastocytoma P815 in a 3 day assay (Fig. 2). Furthermore, if P815 cells were starved for 16 hours in medium lacking Cbl and TCII prior to performing the bioassay, similar Cbl/TCII dependent growth responses were obtained after only 2 days in

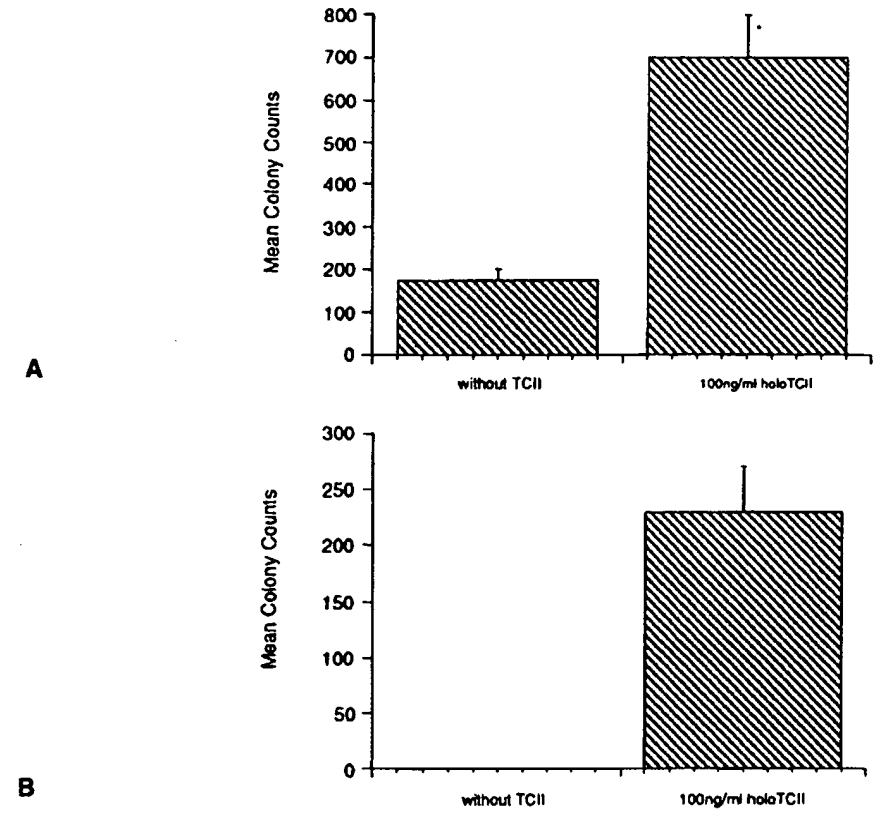


FIGURE 1 *Colony Forming Growth of BW5147 Cells is Dependent on TCII.* BW5147 cells were plated out (1000 cells/plate) in deficient bioassay medium containing 0.3% Bacto agar in the absence or presence of 100ng/ml holoTCII for 7 days. (A) media containing methionine. (B) media lacking methionine. Results are expressed as the mean ± SEM of triplicate determinations.

culture (data not shown). P815 cells display essentially the same dose responses *in vitro* to CN-Cbl (Fig≥ 2A) and recombinant human holoTCII (Fig. 2B) as BW5147 and K562 cells but P815 cells respond in a shorter time span to lack of Cbl/TCII than the other cell lines examined. This is in agreement with the respective proliferative rates of these cell lines under standard *in vitro* conditions (P815 cells have a shorter doubling time of approximately 12 hours whereas the doubling times for BW5147 and K562 cells are approximately 14 hours and 16 hours respectively). Thus, cells which divide more rapidly under standard culture conditions respond to lack of Cbl and/or TCII more rapidly in our bioassay. This is consistent with the previously reported finding that expression of TCII receptors correlates with the proliferative status of the cell.[28] Furthermore, it has been hypothesised that rapidly growing cells, such as leukemic cells, have an increased consumption of Cbl.[29] This may reflect that endogenous Cbl is exhausted more quickly in rapidly dividing cells.

We have adapted the K562 cell line to grow continuously in our specially designed bioassay medium supplemented with 100ng/ml recombinant human holoTCII but lacking folic acid, bovine TCII and Cbl. Cells were passaged in this medium for up to 6 months without appreciable changes in doubling time. Stepwise reduction in the amount of TCII in the culture medium resulted in decreased growth of the adapted K562 cells until no growth was observed with less than 1 ng/ml holoTCII (Fig. 3). These results indicate that holoTCII and therefore Cbl, is an absolute

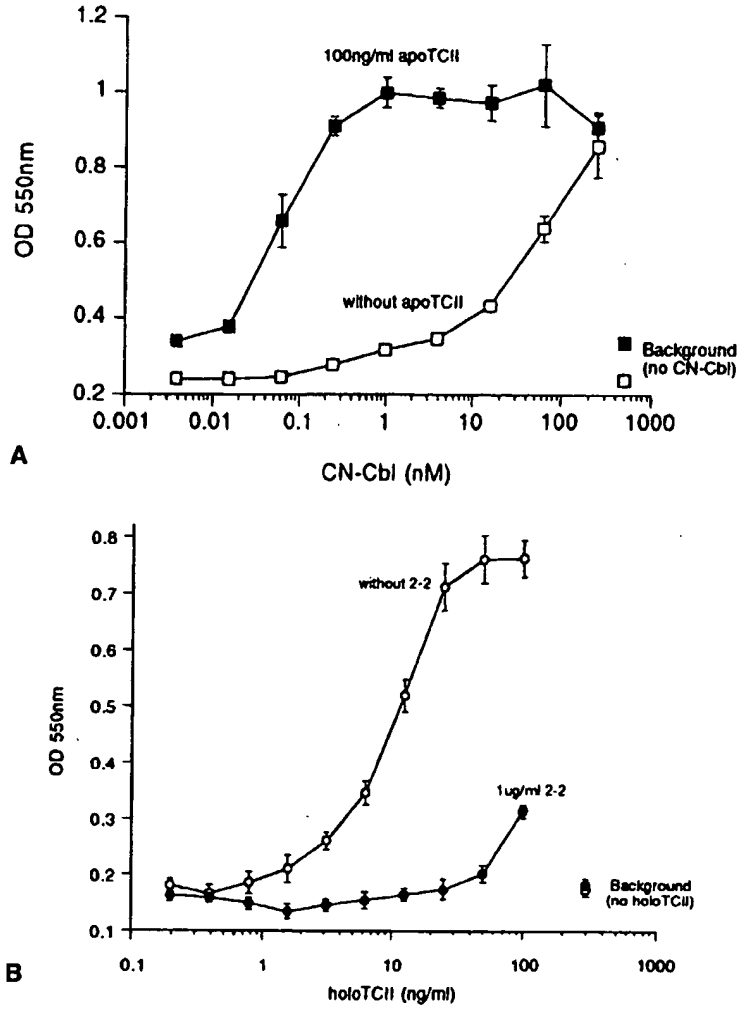


FIGURE 2 *Proliferation of P815 Cells is Dependent on Cbl/TCII.* P815 cells were seeded at 5000 cells/well in bioassay medium containing serial dilutions of CN-Cbl in the absence (open square) and presence (closed square) of 100ng/ml apoTCII (A) and holoTCII in the absence (open circle) and presence (closed circle) of 1ug/ml antibody 2-2 (B) and cultured for 3 days at 37°C. Results are expressed as the mean ± SEM of triplicate determinations.

requirement for leukemic cell growth *in vitro*. Furthermore, we also adapted the BW5147 line to grow in a TCII dependent fashion in another specially designed growth media. For these conditions, the base RPMI was identical to above but the 10% QUSO-treated FCS was replaced with 1% human serum to which 600pM CN-Cbl had been added to fully saturate all available TCII to the holo form without leaving free CN-Cbl. BW5147 cells that had previously been adapted to grow in complete RPMI supplemented with 10% human serum were able to be passaged continuously in the above specialised media for up to 6 months without appreciable changes in doubling time (data not shown).

In general, the propagation of mammalian cells *in vitro* requires that the culture medium contains well defined constituents and is supplemented with a serum source (usually FCS). The system we describe lacks

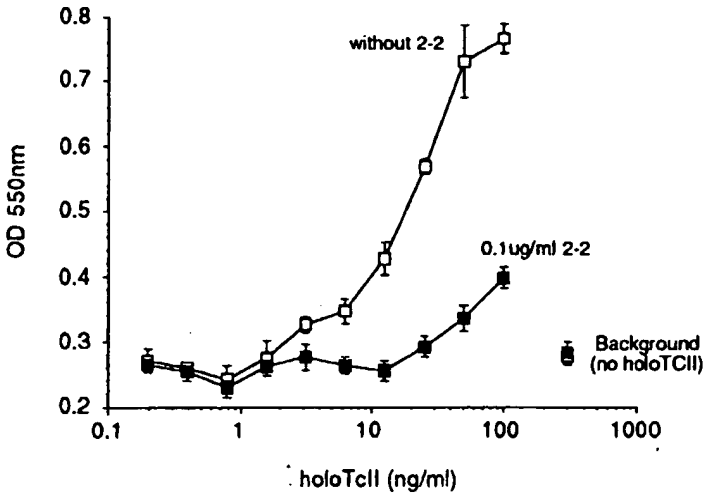


FIGURE 3 *K562 Cells May be Adapted to Require TCII for Proliferation.* K562 cells adapted to grow on holoTCII were seeded at 5000 cells/well in bioassay medium containing serial dilutions of holoTCII in the absence (open square) and presence (closed square) of 0.1ug/ml antibody 2-2 and cultured for 4 days at 37°C. Results are expressed as the mean ± SEM of triplicate determinations.

Cbl, TCII and folic acid which were replaced by metabolites involved in Cbl metabolism to allow Cbl/TCII dependent cell growth. This system has allowed us to determine in various ways the role of TCII in *in vitro* cell growth. We have shown that the bovine TCII in the serum source may be replaced by recombinant human holoTCII in order for optimal *in vitro* cell growth. Also we have shown that a reduced concentration of serum in the culture medium can support cell growth if TCII is converted to the holo form by the addition of Cbl. These results highlight the importance of Cbl and TCII in the growth of leukemic cells and indicate that these could prove to be useful targets for antiproliferative strategies. This may be particularly appropriate for the treatment of AML and CML since serum levels of TCII can be significantly increased in these patients.[130]

Inhibition of TCII Binding to Leukemic Cells by Anti-TCII Antibodies

The anti-TCII antibodies have been previously shown to inhibit delivery of Cbl to cells *in vitro*.[124] To determine if anti-TCII antibodies could block binding of TCII to human leukemic cells, we performed binding assays using K562 cells grown in log phase and human serum saturated with ^{57}Co-Cbl as a source of ^{57}Co-Cbl-TCII. Our results (Fig. 4) indicate that some of the anti-TCII monoclonal antibodies (2–2, 3–11, 4–7) completely inhibit binding of ^{57}Co-Cbl-TCII to K562 cells while other antibodies used at the same concentration resulted in only partial inhibition of ^{57}Co-Cbl-TCII binding. In previous studies we demonstrated that these inhibitory antibodies cross compete for the same epitope on TCII suggesting that they bind to a site on TCII that is in close proximity to the site recognised by the TCII receptor. Antibody (2–6), previously shown to recognise an epitope which is different from the one recognised by antibodies (2–2, 3–11, 4–7), also inhibited binding by 90% (Fig. 4). Several other antibodies (1–9, 5–18, 3–9, 3–5), only partially inhibit binding of TCII to K562 cells (Fig. 4). These results support those previously reported by Quadros et al[24] who showed that antibodies 2–2, 3–11, and 4–7 blocked Cbl uptake by K562 cells and the other anti-TCII antibodies were less effective. As an extra control, 10mM EGTA was included in one point to show that binding of TCII to K562 cells was Ca^{+2} dependent

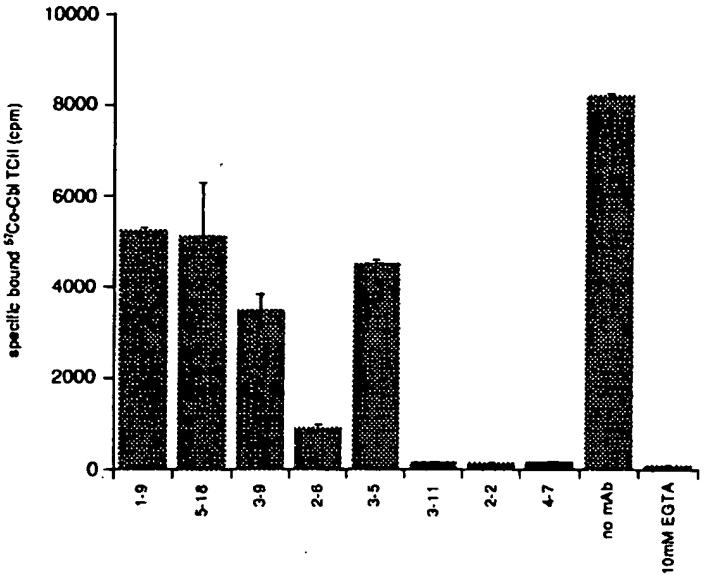


FIGURE 4 *Specifc anti-TCII Monoclonal Antibodies Inhibit Binding of Labelled TCII to K562 Cells.* 10^6 K562 cells in PBS were incubated with ^{57}Co-Cbl labelled human serum for 1 hour at 37°C in the presence of 10ug/ml anti-TCII antibodies, 10mM EGTA, or no additions. Cell pellets were assessed for γ radiation as described. Results are expressed as the mean ± SEM of duplicate determinations.

and that no ^{57}Co-Cbl was taken up by the cells under the binding conditions used.

Inhibition of Cell Growth by Anti-TCII Antibodies

Our earlier work showed that anti-TCII monoclonal antibodies can inhibit the growth of K562 and BW5147 cells *in vitro*.[25] Here we show that P815 cell growth *in vitro* is inhibited by these antibodies (Figs. 2B and 5) with a similar dose response pattern as was previously demonstrated for BW5147 and K562 cells. When K562 cells adapted to growth in recombinant holoTCII (Fig. 3) were grown in the presence of 0.1ug/ml of the anti-TCII monoclonal antibody 2-2, TCII dependent cell growth was strongly reduced even in excess TCII (Fig. 3).

In the case of BW5147 cells that had been adapted for TCII dependent growth in human serum, similar results were observed. Addition of antibody (2-2) to the growth medium inhibited cell proliferation while antibody (1–9) had no effect at the concentrations tested (Fig. 6). Taken together, these results show that regardless of; i) the cell type; ii) the source of human TCII; and iii) conditions for TCII dependent proliferation; similar patterns of inhibition by the anti-TCII antibodies were observed.

Others have so far not been able to demonstrate inhibition of cell growth *in vitro* by anti-TCII antibodies, although these reagents have existed for some time. However, a report in 1995 by Bose *et al.*[31] described the production of human TCII receptor specific rabbit antiserum. Interestingly, the rabbits injected with purified human TCII receptor for production of antiserum, failed to thrive although tissue or organ damage was not apparent.[32] Further analysis showed that the circulating antibodies to human TCII receptor bound to and inactivated the rabbit TCII receptor, resulting in severe Cbl deficiency and the failure of the rabbits to thrive. This supports our data and indicates that targeting Cbl at the level of the binding protein and cell surface receptor may prove to be useful in antiproliferative strategies.

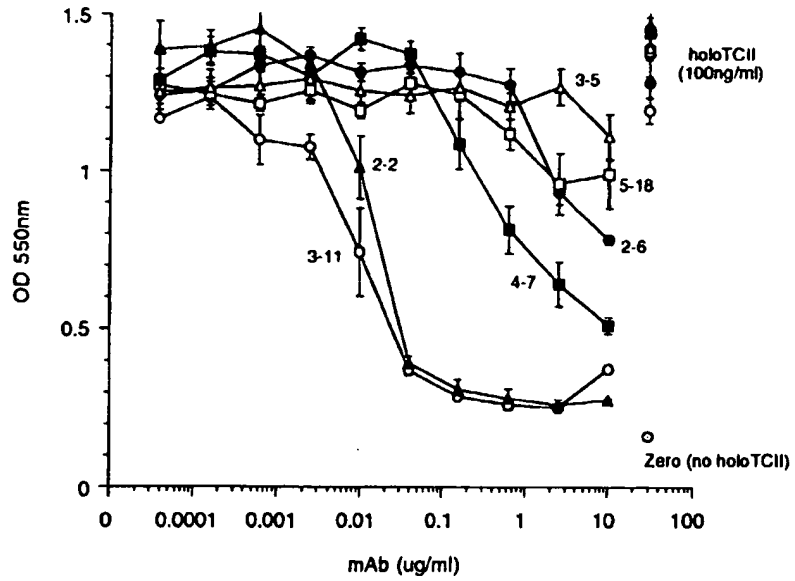


FIGURE 5 *P815 Cell Proliferation is Inhibited by anti-TCII Monoclonal Antibodies.* P815 cells were seeded at 5000 cells/well in bioassay medium containing serial dilutions of anti-TCII antibodies [3–5 (open triangle), 5–18 (open square), 2–6 (closed circle), 4–7 (closed square), 2-2 (closed triangle), 3–11 (open circle)] in the presence of 100ng/ml holoTCII and cultured for 3 days at 37°C. Results are expressed as the mean ± SEM of triplicate determinations.

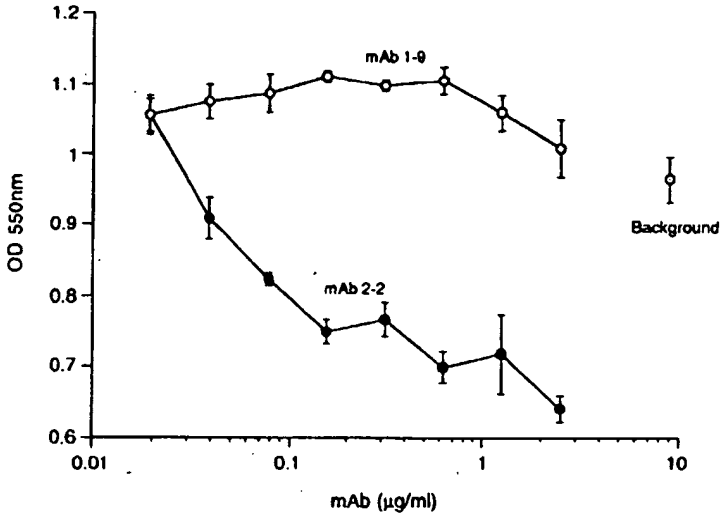


FIGURE 6 *BW5147 Cells may be Adapted to Proliferate Dependently on TCII.* BW5147 cells were seeded at 5000 cells/well in bioassay medium containing 1% Cbl-saturated human serum in the presence of serial dilutions of anti-TCII antibodies 1–9 (open circle) and 2-2 (closed circle) and cultured for 4 days at 37°C. Results are expressed as the mean ± SEM of triplicate determinations.

References

[1] Hall, C. A. and Finkler, A. E. (1966). Function of transcobalamin II: A B12 binding protein in human plasma. Proc Soc Exp Biol Med 123:55–58

[2] Quadros, E. V., Rothenberg, S. P., Pan, Y. E. and Stein, S. (1986) Purification and molecular charcterization of human transcobalamin II. J Biol Chem 264:15455–15460

[3] Youngdahl-Turner, P., Mellman, I. S., Allen, R. H. and Rosenberg, L. (1979) Protein mediated vitamin uptake. Adsorptive endocytosis of the transcobalamin II-cobalamin complex by cultured human fibroblasts. Exp Cell Res 118:127–134

[4] Bose, S., Feix, J., Seetharam, S. and Seetharam, B. (1996a) Dimerization of transcobalamin II receptor. J Biol Chem 271:11718–11725

[5] Quereshi, A. A., Rosenblatt, D. S. and Cooper, B. A. (1994) Inherited disorders of cobalmin metabolism. Crit Rev Oncol Hematol 17:133–151

[6] Herbert, V. and Zalusky, R. (1962) Interrelationships of vitamin B12 and folic acid metabolism: Folic acid clearance studies. J Clin Invest 41:1263–1276

[7] Lassen, H. C. A., Henriksen, E., Neukirch, F. and Kristensen, H. S. (1956) Treatment of tetanus. Severe bone marrow depression after prolonged nitrous-oxide anaesthesia. Lancet 1:527–531

[8] Kieler, J. (1957) The cytotoxic effect of nitrous oxide at different oxygen tensions. Acta Pharmacol. Toxicol (Copenh) 13:301–306

[9] Fink, B. R. (1966) Carcinostatic action of nitrous oxide in mice. Anesthesiology 27:214–219

[10] Parbrook, G. D. (1967) Experimental studies into the effect of nitrous oxide on tumor cell growth. Br. J. Anaesth. 39:549–556

[11] Deacon, R., Lumb, M., Derry, J., Channarin, I., Barbara, M., Halsay, M. and Nunn, J. (1980) Inactivation of methionine synthase by nitrous oxide. Eur. J. Biochem. 104:419–422

[12] Huennekens, F. M., DiGirolamo, P. M., Fujii, K., Jacobsen, D. W. and Vitols, K. S. (1976) In: Weber G. (ed): Adv. Enz. Regul. Oxford: Pergamon vol 14, p187

[13] Lassen, H. C. A. and Kristensen, H. S. (1959) Remission of chronic myeloid leukemia following prolonged nitrous oxide inhalation. Dan. Med. Bull. 6:252–255

[14] Eastwood, D. W., Green, C. D., Lambdin, M. A. and Gardner, R. (1963) Effect of nitrous oxide on the white-cell count in leukemia. New Eng J Med 268:297–299

[15] Kroes, A. C. M., Lindemans, J., Hagenbeek, A. and Abels, J. (1984a) Nitrous oxide reduces growth of experimental rat leukemia. Leukemia Res. 8:441–448

[16] Konno, M., Kirikae, T., Suzuki, K. S., Yoshida, M., Mori, K. J. and Wakusawa, R. (1988) Increased lethality and delay in the recovery of hemopoietic stem cells after irradiation in mice exposed to nitrous oxide. Acta. Anaesthesiol. Scand. 32:213–217

[17] Ermens, A. A. M., Vink, N., Schoester, M., van Lom, K., Lindemans, J. and Abels, J. (1989) Nitrous oxide selectively reduces the proliferation of the malignant cells in experimental rat leukemia. Cancer Letters 45:123–128

[18] Warren, D. J., Christensen, B. and Slørdal, L. (1993) Effect of nitrous oxide on haematopoiesis *in vitro*: Biochemical and functional features. Pharmacol. Toxicol. 72:69–72

[19] Kroes, A. C. M., Lindemans, J. and Abels, J. (1984b) Synergistic growth inhibiting effect of nitrous oxide and cycloleucine in experimental rat leukemia. Br J Cancer 50:793–800

[20] Kroes, A. C. M., Lindemans, J., Schoester, M. and Abels, J. (1986) Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (vitamin B12) by nitrous oxide. Cancer Chemother Pharmacol 17:114–20

[21] Ermens, A. A. M., Schoester, M., Lindemans, J. and Abels, J. (1991) Effect of nitrous oxide and methotrexate on folate coenzyme pools of blast cells from leukemia patients. Leuk Res 15:165–171

[22] Walker, P. R., Smith, B., Carson, C., LeBlanc, J., Sikorska, M., Woodhouse, C. S. and Morgan, A. C. (1997) Induction of apoptosis in neoplastic cells by depletion of vitamin B12. Cell Death Diff 4, 233–241.

[23] Quadros, E. V., Sai, P. and Rothenberg, S. P. (1993) Functional human transcobalamin II isoproteins are secreted by insect cells using the baculovirus expression system. Blood 81, 1239–1245

[24] Quadros, E. V., Rothenberg, S. P. and McLoughlin, P. (1996) Characterization of monoclonal antibodies to epitopes of human transcobalamin II. Biochem Biophys Res Comm 222, 149–154

[25] McLean, G. R., Quadros, E. V., Rothenberg, S. P., Morgan, A. C., Schrader, J. W. and Ziltener, H. J. (1997) Antibodies to transcobalamin II block *in vitro* proliferation of leukemic cells. Blood 89, 235–242

[26] Mossman, T. (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65, 55–64

[27] Hansen, M. B., Nielsen, S. E. and Berg, K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119, 203–210

[28] Amagasaki, T., Green, R. and Jacobson, D. W. (1990) Expression of transcobalamin II receptors by human leukemia K562 and HL-60 cells. Blood 76, 1380–1386

[29] Tanaka, N. (1992) Vitamin B12 (cobalamin) contents of serum and organs in various diseases and its clinical significance. J. Nutr. Sci. Vitaminol. (Tokyo). Spec No: 104–109

[30] Neale, G. (1990) B12 binding proteins. Gut 31:59–63

[31] Bose, S., Seetharam, S. and Seetharam, B. (1995) Membrane expression and interactions of human transcobalamin II receptor. J Biol Chem 270, 8152–8157

[32] Bose, S., Komorowski, R., Seetharam, S., Gilfix, B., Rosenblatt, D. S. and Seetharam, B. (1996b) *In vitro* and *in vivo* inactivation of transcobalamin II receptor by its antiserum. J Biol Chem 271, 4195–4200

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

67097883 CA: 67(21)97883j JOURNAL
Character of antagonism of cobalamin monocarboxylic acid toward some analogs of cobalamin
AUTHOR(S): Simon, Agoston
LOCATION: Fabrik Chem. Pharm. Prod. Chinoin, Budapest, Hung.
JOURNAL: Zentralbl. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt. 2: Allg., Landwirtsch. Tech. Mikrobiol. DATE: 1967 VOLUME: 121 NUMBER: 2
PAGES: 199-205 CODEN: ZBPIA9 LANGUAGE: German
SECTION:
CA808000 Microbial Biochemistry
IDENTIFIERS: VITAMIN B12 ANTAGONISTS, COBALAMINS MONOCARBOXYLIC ACID, CYANOCOBALAMIN ANTAGONISTS
DESCRIPTORS:

9068049

Remarks:

4876622 10/5/30

STIC Use Only Accession Number: 397777

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

[Aus der Fabrik für Chemische und Pharmazeutische Produkte „Chinoin" Budapest]

Über den Charakter des Antagonismus der Cobalaminmonocarbonsäure gegenüber einigen Analogen des Cobalamins

A. Simon

Mit einer Abbildung im Text

Für den Davis' Stamm *E. coli* 113-3 ist die Cobalaminmonocarbonsäure ein kompetitiver Antagonist des Cyanocobalamins (1). Wird dieser Antagonismus mit dem Agardiffusionstest untersucht, findet man folgende Resultate:

1. Die Cyanocobalaminmonocarbonsäure antagonisiert vollständig den Effekt des Cyanocobalamins in 40facher Konzentration. Dieser Faktor 40 ist eine konstante, von der Konzentration des Cyanocobalamins unabhängige Zahl.

2. Wird zu einer bestimmten Lösung von Cyanocobalamin Cobalaminmonocarbonsäure in steigenden Konzentrationen zugegeben und werden die so erhaltenen Lösungen auf einer für biologische B_{12}-Vitaminbestimmung vorbereiteten Agarplatte untersucht, findet man bis zu einer etwa 5fachen Konzentration der Monocarbonsäure keinen sichtbaren Unterschied. Im Falle einer 10 oder 20fachen Konzentration der Monocarbonsäure wird die Wuchszone etwas (um 1—2 mm) größer und verschwommener. Ist die Konzentration noch höher, so verschwindet die Wuchszone oder bildet sich eine kaum wahrnehmbare Opazität aus (2).

3. Auf einer Agarplatte, die sehr kleine Mengen Vitamin B_{12} enthält, wirkt die Cobalaminmonocarbonsäure wie ein Antibiotikum und ruft eine regelrechte Inhibitionszone hervor (3).

Im Besitz dieser Daten schien es uns interessant, den antagonistischen Effekt der Cyanocobalaminmonocarbonsäure gegenüber gewissen Cobalamin-Analogen zu untersuchen und zu charakterisieren.

Methodik und verwendete Substanzen

Vitamin B_{12}. Es wurde das kristallisierte Produkt der Firma Chinoin verwendet. Das Produkt wurde zweimal umkristallisiert und die Reinheit durch Papierchromatographie und Elektrophorese kontrolliert.

Cobinamid (Faktor B). Das Präparat wurde folgendermaßen dargestellt: es wurde bei 26 °C eine Woche lang, *Propionibacterium shermanii* auf einem Nährboden gezüchtet, der 0,25% Mais-Extrakt (Corn-steep liquor)-Trockensubstanz, 2% Glukose, 0,5% $(NH_4)_2SO_4$ sowie

$CaCO_3$, Co und Spurenelemente enthielt. Der pH-Wert wurde durch Zugabe von NH_4OH zwischen 6 und 7 gehalten. Nach einer Woche wurde die Kultur zentrifugiert, das Sediment mit Wasser ausgewaschen, wieder zentrifugiert und die Bakterienmasse mit 60%igem Methanol extrahiert. Die durch Zentrifugieren geklärte methanolische Lösung wurde unter vermindertem Druck eingeengt. Die so erhaltene Lösung gibt nach Zugabe von KCN eine violette Färbung und enthält außer Cobinamid keine andere Cobalamin-Analogen, wie es durch Chromatographie im System Sec. Butanol-Wasser bestätigt wurde. Die Konzentration der Lösung wurde der biologischen Aktivität entsprechend eingestellt (auf der Agarplatte gemessene scheinbare Aktivität).

Faktor III (5-hydroxybenzimidazol-Cobalamin). Ein kommerzielles, unreines Präparat wurde im System Sec. Butanol-Wasser chromatographiert. Es enthielt 90% Faktor III, 8,2% Cyanocobalamin und 1,8% andere Substanzen. Es wurde mit dem chromatographisch gereinigten, isolierten Faktor III gearbeitet.

Cobalaminmonocarbonsäure. Die Substanz wurde durch Hydrolyse von Vitamin B_{12} hergestellt (4).

Der Antagonismus wurde auf nach Tschaikowskaja und Druzinina hergestellten Agarplatten untersucht (5).

Ergebnisse

1. Antagonismus der Cobalaminmonocarbonsäure mit Cobinamid und Faktor III.

Es wurde der Antagonismus zwischen Cobalaminmonocarbonsäure und Kobinamid bzw. Faktor III. untersucht. In die Agarplatten (5) wurden Löcher gebohrt, und in die Löcher wurde je ein Tropfen von den verschiedenen Lösungen eingetragen, die die antagonistischen Substanzen in verschiedenen Verhältnissen enthielten. Die Ergebnisse sind in Tabelle I zusammengefaßt.

Tabelle I

Beschreibung der Zonen, die durch verschiedene Mischungen von Cobalaminmonocarbonsäure und der Faktoren entstanden

Verhältnis der Konzentrationen der Cobalaminmonocarbonsäure und des Faktors	Angewendeter Faktor: Cobinamid — Beschreibung der Zonen	Angewendeter Faktor: Faktor III — Beschreibung der Zonen
0,	25,5	25,5
3,75	25,5	25,5 V.
7,5	26,5	27,0 R.
15,0	27,0 R.	— H., R.
30,0	— H., R.	— H., R.
60,0	Keine Zone	Keine Zone

Erklärung: Die Zahlen bedeuten den Durchmesser der Zonen in mm. Wo keine Zahlen angegeben, war die Zone verschwommen und unmeßbar.
V. = Zone mit verschwommenem Rand.
R. = Ringförmige Wuchszone mit einer Inhibitionszone im Zentrum.
H. = Die ganze Zone ist verschwommen und kaum sichtbar.

Aus den in der Tabelle I beschriebenen Ergebnissen kann man folgende Schlüsse ziehen:

1. Die Cobalaminmonocarbonsäure ist Antagonist nicht nur des Cyanocobalamins, sondern auch der untersuchten Faktoren. Man bekommt eine komplette Hemmung, wenn der Quotient Antagonist/Faktor bei oder über 60 liegt. (Bei Cobinamid wird dabei die virtuelle, an der Agarplatte gemessene falsche Konzentration als „Konzentration" betrachtet.)

2. Der Antagonismus zwischen Cobalaminmonocarbonsäure und den untersuchten Analogen ist vom Antagonismus zwischen Cobalaminmonocarbonsäure und Vitamin B_{12} verschieden.

Bei der Anwendung von verschiedenen Mischungen von Vitamin B_{12} und Cobalaminmonocarbonsäure bekommt man nämlich homogene Wuchszonen. Bei der stufenweisen Erhöhung der relativen Cobalaminmonocarbonsäure-Konzentration werden die Zonen um 1—2 mm größer, dabei werden sie aber verschwommener, bis sie am Ende ganz verschwinden.

Bei den untersuchten Faktoren wurden bei gewissen Konzentrationsverhältnissen in der Mitte der Wuchszone ganz gut definierte Hemmringe beobachtet. Bei der weiteren Erhöhung der relativen Konzentration des Antagonisten verschwindet allmählich die ganze Wuchszone, da die Hemmzone immer größer und die Wuchszone immer verschwommener wird.

In diesen Fällen verursacht die Cobalaminmonocarbonsäure eine zentrale Hemmzone, die denen ähnelt, welche durch eine Mischung von Vitamin B_{12} und Oxytetracyclin oder andere, gegen *E. coli* 113-3 aktive Antibiotika ausgelöst werden.

II. Durch Vitamin B_{12}, Cobalaminmonocarbonsäure und Cobinamid bzw. Faktor III ausgelöste Ringphänomene.

In einem Versuch wurden Cyanocobalamin, Cobalaminmonocarbonsäure und Cobinamid zusammen auf die Agarplatte (5) in folgenden Konzentrationen aufgetragen:

Cyanocobalamin	0.5 μg/ml
Cobalaminmonocarbonsäure	10.0 μg/ml
Cobinamid (virtuelle Aktivität) . .	0.5 μg/ml

In diesem Falle, in gewisser Übereinstimmung mit dem vorigen Experiment, wurde eine dreifache Zone erhalten: eine innere Wuchszone, ein äußerer Wuchsring und zwischen den beiden ein Hemmring (siehe Abb. 1).

Wenn das ungereinigte Präparat des Faktors III in einer zweifachen Verdünnungsreihe mit immer derselben Menge von Cobalaminmonocarbonsäure auf die Agarplatte aufgetragen worden war, wurde ein ähnliches Ergebnis erhalten. Wurden ungereinigte Vitamin B_{12}-Lösungen in verschiedenen Verhältnissen mit Cobalaminmonocarbonsäure zusammengemischt auf die Agarplatte (5) aufgetragen, wurden ebenfalls solche Ringerscheinungen beobachtet.

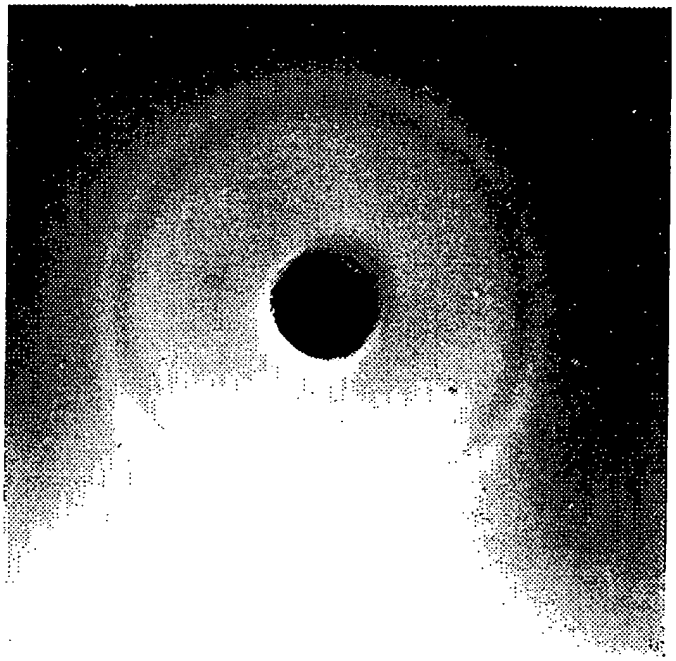


Abb. 1. Trennung von Cyanocobalamin und Cobinamid durch Anwendung von Cobalaminmonocarbonsäure.
Der Hemmring entspricht der Cobalaminmonocarbonsäure, die innere Wuchszone dem Vitamin B_{12} und der äußere Wuchsring dem Cobinamid. 2,3fache Vergrößerung

Besprechung der Ergebnisse

Fantes und Mitarb. (6). Simon (7, 9) und auch andere Autoren haben bewiesen, daß bei der Agardiffusionsmethode des Cyanocobalamins und seiner Analogen keine freie Diffusion auf der mit Bakterien beimpften Agarplatte stattfindet, sondern die genannten Substanzen durch die Mikroorganismen absorbiert werden und der Umfang der Wuchszonen außer der Konzentration der auf die Agarplatte aufgetragenen Wuchsstoffe der Vitamin B_{12}-Reihe auch von der Intensität der Bindung an die Bakterien abhängig ist. Beispielsweise ist es eine wohl bekannte Tatsache, daß das Cobinamid bei dem Agardiffusionstest etwa fünfmal aktiver zu sein scheint, als bei der Bestimmung durch die turbidimetrische Methode (Ford, 8), was dadurch zu erklären ist, daß dieser Stoff sich schwächer an die Bakterien bindet als das Cyanocobalamin.

Die hier beschriebenen Versuche zeigen, daß das Cyanocobalamin fester durch die Cobalaminmonocarbonsäure gebunden wird. Deshalb diffundiert ein wesentlicher Teil der letzteren Substanz in die außerhalb der durch das Cyanocobalamin hervorgerufenen Wuchszone. Unter normalen Bedingungen wird jedoch diese Tatsache nicht wahrgenommen, da es nichts gibt, was die Anwesenheit dieses

Hemmstoffes auf der Agarplatte indizieren würde. Doch wurde vom Verfasser in einer vorigen Publikation (2) ein Versuch beschrieben, bei dem diese Indikation möglich war. Es wurden geeignete Mischungen von Cyanocobalamin und Cobalaminmonocarbonsäure auf eine Agarplatte (5) in zueinander naheliegenden Punkten aufgetragen. Die sich ausbildenden Zonen flossen nicht zusammen, sondern wurden deformiert, fast viereckig, durch schmale, unbewachsene Streifen voneinander getrennt. So wird das Vorhandensein des antagonistischen Faktors durch die nebeneinander ausgebildeten Wuchszonen am Rande derselben indiziert.

Die hier beschriebenen Versuche sprechen auch dafür, daß die Cobalaminmonocarbonsäure fester von den Testorganismen gebunden wird als die untersuchten Cobalamin-Analogen (Cobinamid, Faktor III). Sie wird durch die Bakterien gebunden, und deshalb fällt ihre Konzentration bei der Diffusion schneller ab als die Konzentration der erwähnten Faktoren. So verschieben sich die Konzentrationsverhältnisse am Rande der Zone zugunsten der letzteren, und es bildet sich eine, durch einen Wuchsring umkreiste, Hemmzone aus.

Aus diesen Tatsachen ergibt sich die Verteilung der Hauptmengen der verschiedenen Substanzen in der Ringerscheinung des II. Versuches. Die Reihenfolge ist vom Mittelpunkt nach dem Rand: Cyanocobalamin, Cobalaminmonocarbonsäure, Cobinamid bzw. Faktor III.

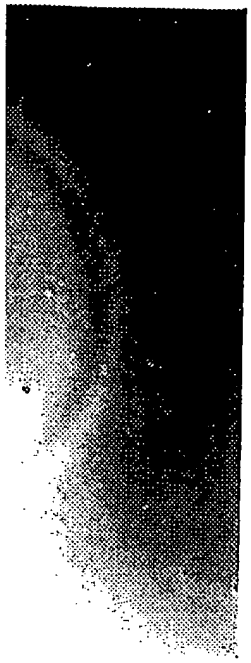
Dieser Versuch stellt eine chromatographische Trennung der drei Faktoren dar. Da aber diese Trennung mit Hilfe der auf die Agarplatte geimpften Bakterien zustande kommt, wobei die Bakterien durch die selektive Bindung der einzelnen Substanzen eine aktive Rolle spielen, kann dieser Prozeß Biochromatographie genannt werden.

Diese einfache Methode ist für die Untersuchung von unreinen Präparaten vom Vitamin B_{12} (Premix etc.) geeignet. Wird nämlich die Aktivität des Präparates mit dem Agardiffusionstest gemessen, wird dann die 20fache Menge der bestimmten Aktivität von Cobalaminmonokarbonsäure zugegeben und wird die Mischlösung auf eine andere Agarplatte (5) aufgetragen, so bilden sich, wenn es sich um reines Vitamin B_{12} handelt, homogene Wuchszonen aus. Es treten aber, wenn beträchtliche Mengen von Faktoren beigemischt sind, Ringphänomene auf. Dieses Auftreten der Ringe beweist, daß Faktoren im Präparat vorhanden sind.

Nach den Literaturangaben soll der Faktor III dieselbe biologische Wirkung besitzen wie Vitamin B_{12}. Nun haben die obigen Versuche einen, wenn auch nicht bedeutenden Unterschied zwischen dem biologischen Verhalten der zwei Substanzen aufgedeckt; sie benehmen sich im Agardiffusionstest mit *E. coli* 113-3 nicht identisch, der Faktor III wird nämlich schwächer durch die Bakterien gebunden. Es bleibt die Frage offen, ob Faktor III auch durch die menschlichen Zellen oder Serumeiweiße schwächer gebunden wird als Cyanocobalamin.

Zusammenfassung

1. Es wurde festgestellt, daß die Cobalaminmonocarbonsäure ein Antagonist nicht nur des Vitamins B_{12}, sondern auch des Cobinamids und des Faktors III ist. Sie hemmt vollständig den auf *E. coli* 113-3 ausgeübten Wuchseffekt dieser Vitamin B_{12}-Analogen in 40 bis 60facher Konzentration.



durch Anwendung von Cobalamin-

carbonsäure, die innere Wuchszone
1 Cobinamid. 2,3fache Vergrößerung

andere Autoren haben bewiesen,
-balamins und seiner Analogen
mpften Agarplatte stattfindet,
roorganismen absorbiert werden
ntration der auf die Agarplatte
e auch von der Intensität der
weise ist es eine wohl bekannte
sionstest etwa fünfmal aktiver
rbidimetrische Methode (Ford.
ich schwächer an die Bakterien

as Cyanocobalamin fester durch
Deshalb diffundiert ein wesent-
der durch das Cyanocobalamin
edingungen wird jedoch diese
t, was die Anwesenheit dieses

2. Der antagonistische Effekt hat zwei verschiedene Formen im Agartest:

a) Gegen Vitamin B_{12} kommt der antagonistische Effekt durch die ganze Wuchszone fast gleichmäßig zum Ausdruck.

b) Gegen Cobinamid und Faktor III wirkend, erzeugt die Cobalaminmonocarbonsäure eine Hemmzone im Zentrum der Wuchszone. Diese letztere wird dadurch ringförmig.

3. Wird Cyanocobalamin, ein Cobalamin-Analog vom Typ b) und Cobalaminmonocarbonsäure in geeigneten Konzentrationsverhältnissen auf die Agarplatte aufgetragen (die geeigneten Verhältnisse können durch eine Verdünnungsreihe erhalten werden), dann bildet sich ein Hemmring zwischen zwei Wuchszonen aus; das heißt, mit Hilfe der Cobalaminmonocarbonsäure können die Faktoren des Typs b) vom Vitamin B_{12} getrennt werden. Dieses Trennungsverfahren kann — da es sich um eine selektive Bindung an lebende Bakterien handelt — Biochromatographie genannt werden. Dieses Verfahren ist unter Umständen geeignet, die Anwesenheit von gewissen Faktoren neben Vitamin B_{12} schnell und einfach festzustellen.

4. Es wurde gezeigt, daß Vitamin B_{12} und Faktor III sich hinsichtlich ihrer Antagonismen gegen Cobalaminmonocarbonsäure im Agartest nicht gleich verhalten, das heißt, Faktor III ist in biologischer Hinsicht kein ganz vollwertiges Substitut für Cyanocobalamin.

Summary

1. It is demonstrated that Cobalaminmonocarboxilic acid is an antagonist not only of Vitamin B_{12}, but also of Cobinamid and of factor III. — It inhibits completely the growth promoting effect of the mentioned analogues of Vitamin B_{12} on the strain *E. coli* 113-3 in 60-fold or higher concentrations.

2. The antagonistic effect may have two different forms of manifestation when tested on the agar-plate;

a) In the case of Vitamin B_{12} the antagonistic effect is expressed by an uniforme faintness of the zone and by indefinite edges.

b) In the case of Cobinamid and Factor III, Cobalamine-monocarboxilic acid produces a zone of inhibition in the center of the zone of growth. So this latter gets the form of a ring.

3. When applying on the agar plate prepared for the test of Vitamin B_{12} a mixture of Cyanocobalamin, Cobalaminmonocarboxilic acid and a factor type b) in adequate concentrations (these concentrations may be obtained by a series of dilutions), one gets a ring of inhibition between two zones of exhibition. So, using Cobalaminmonocarboxilic acid, it is possible to separate the factors type b) from Vitamin B_{12}. As this separation is due to the active and selective absorption of the mentionated compounds by living bacteria, the name of Biochromatography is proposed for this method. — This rapid and simple method is recommended for demonstrating the presence of various factors beside Vitamin B_{12}.

4. It is demonstrated that Vitamin B_{12} and factor III have not the very same biological effects, as formerly it has been believed. Namely there is some difference between them concerning their antagonism to Cobalamin-monocarboxilic acid. So, factor III may not be considered as an absolutely equivalent substitute for Cyanocobalamin.

schiedene Formen im Agartest:
nistische Effekt durch die ganze

und, erzeugt die Cobalaminmono-
Wuchszone. Diese letztere wird

alog vom Typ b) und Cobalamin-
verhältnissen auf die Agarplatte
en durch eine Verdünnungsreihe
g zwischen zwei Wuchszonen aus;
nsäure können die Faktoren des
eses Trennungsverfahren kann —
Bakterien handelt — Biochro-
rfahren ist unter Umständen ge-
eben Vitamin B_{12} schnell und ein-

Faktor III sich hinsichtlich ihrer
e im Agartest nicht gleich ver-
Hinsicht kein ganz vollwertiges

carboxilic acid is an antagonist
and of factor III. — It inhibits
ntioned analogues of Vitamin B_{12}
concentrations.
different forms of manifestation

tic effect is expressed by an uni-
dges.
Cobalamine-monocarboxilic acid
e zone of growth. So this latter

red for the test of Vitamin B_{12}
boxilic acid and a factor type b
may be obtained by a series of
vo zones of exhibition. So, using
eparate the factors type b) from
ctive and selective absorption of
ne name of Biochromato-
rapid and simple method is re-
ious factors beside Vitamin B_{12}.
l factor III have not the very
believed. Namely there is some
gonism to Cobalamin-monocar-
red as an absolutely equivalent

Literatur

1. Kelemen, Á. M. und A. Simon, Acta Microbiol. Acad. Sci. Hung. **8**, 223 (1961). — 2. Kelemen, Á. M. und A. Simon, Ibid. **8**, 237 (1961). — 3. Kelemen, Á. M. und A. Simon, Antibiot. Congr. 1964. Praga. Abstr. of papers, 141. — 4. Ungarisches Patent GO-906. — 5. Tschaikowskaja, S. M. und E. N. Druzinina, Mikrobiologija **26**, 609 (1957). — 6. Fantes, K. H., u. Mitarb., Biochim. et Biophys. Acta **20**, 397 (1956). — 7. Simon, A., Antibiotiki **4**, No. 1, 108 (1959). — 8. Ford, J. E., Brit. J. Nutr. **7**, 299 (1953). — 9. Simon, A., Zbl. Bakt. II **120**, 10 (1966).

Anschrift des Verfassers:

Augustin Simon, Budapest II, Vöröshadsereg u. 33 (Ungarn).

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name: Patricia Duffy | Org or A.U.: 1645 | Phone: 305-7555

Serial Number: 09/654,114 | Req. Date: ASAP | Date:

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Miasishcheva ed
Journal/Book Title:	Voprosy meditsinskoi khimii
Article Title:	
Volume (Issue):	~~25~~ 23(5)
Pages:	622-9
Year of Publication:	1977 (Sept-Oct)
Publisher:	
Remarks:	

7068041 #05/30

339529

STIC Use Only Accession Number: 97780

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

ЛИТЕРАТУРА

1. Meister A. Biochemistry of the Amino Acids. V. 2. New York, 1965, p. 622.— 2. Novak E. K., Phillips A. W. — «J. Bact.», 1974, v. 117, p. 593—600. — 3. Campbell H. A., Mashburn L. T. — «Biochemistry (Wash.)», 1969, v. 8, p. 3768. — 4. Tosa Tetsuya, Sano Ryujiro, Yamamoto Kozo. — «Biochemistry (Wash.)», 1962, v. 2, p. 216. — 5. Roberts J., Holcenberg J. S., Dolowy W. S. — «J. biol. Chem.», 1972, v. 247, p. 84. — 6. Holcenberg J. S., Teller T. C., Roberts J. et a.— Ibid., 1972, v. 247, p. 7750. — 7. Ramadan M. E. A., El Asmal E., Greenberg D. M. — «Arch. Biochem.», 1964, v. 108, p. 150. — 8. Katsumata H., Katsumata R., Abe T. — «Biochim. biophys. Acta», 1972, v. 289, p. 405. — 9. Мардашев С. Р., Николаев А. Я., Коваленко Н. А. и др. — «Вопр. мед. химии», 1975, № 1, с. 29. — 10. Davis B. J., et a. — «Ann. N. Y. Acad. Sci.», 1964, v. 121, p. 321. — 11. Коваленко Н. А., Козлов Е. А., Герасимова А. В. — «Биохимия», 1970, т. 35, с. 670. — 12. Мардашев С. Р., Лестровая Н. Н. — «Вопр. мед. химии», 1949, № 1, с. 203. — 13. Пасхина Т. С. — В кн.: Современные методы в биохимии. Т. 1. М., 1964, с. 162. — 14. Kjaer A., Larsen P. O. — «Acta chem. scand.», 1959, v. 13, p. 1565. — 15. Ohnuma T., Bergel F., Brey R. C. — «Biochem. J.», 1967, v. 103, p. 238. — 16. Howard J. B., Carpenter F. H. — «J. biol. Chem.», 1972, v. 247, p. 1020. — 17. Козлов Е. А., Коваленко Н. А. — «Успехи биол. химии». Т. 13, 1974, с. 214. — 18. Herrmann V., Röhm K. H., Schneider F. — «FEBS Letters», 1974, v. 39, p. 214. — 19. Соколов Н. Н., Николаев А. Я., Мардашев С. Р. — «Микробиология», 1971, т. 40, с. 631.

Поступила 7/VI 1976 г.

SOME DATA ON SUBSTRATE SPECIFICITY, INHIBITORS AND KINETICS OF DEAMIDASE AG (ASPARAGINASE-GLUTAMINASE) FROM PSEUDOMONAS FLUORESCENS AG

N. A. Kovalenko, T. A. Tsvetkova, A. Ya. Nikolaev

Chair of Biochemistry, I. M. Sechenov Medical School Laboratory of Enzymology, Academy of Medical Sciences of the USSR, Moscow

Deamidase AG (asparaginase-glutaminase) from Pseudomonas fluorescens AG was shown to hydrolyze l-glutamine and l-asparagine highly effectively. Besides, the enzyme exhibited the rather high rate of deamidation of D-asparagine and D-glutamine (70% and 100%, respectively), N_{α}-butyl asparagine (63%) and among peptides — of glycyl-L-asparagine (40%). L-glutamic acid γ-methyl ester was hydrolyzed only slightly (5%). Effect of several substrate analogues on the deamidase AG activity was studied as well. Albiciine (α-amino-β-ureide propionic acid) proved to be the strongest inhibitor (100%). β-Methyl aspartic acid, S-carbamoyl cysteine, α-ketoglutaric acid showed the slight inhibitory effect (20%). Amount of active centres per enzyme molecule was estimated by means of ^{14}C-albiciine. Deamidase AG had apparently only one active centre. In estimation of relationship between the rate of reaction and substrate (L-asparagine) concentration, the reaction was found to follow Michaelis-Menten kinetics, $K_m = 4.5 \cdot 10^{-4}$ M.

УДК 612.014.3:612.6.015.641.61

Н. В. Мясищева, О. Д. Голенко, Л. Е. Кузнецова, М. О. Раушенбах, И. П. Рудакова, Е. М. Тачкова, А. М. Юркевич

ВЛИЯНИЕ МЕТИЛКОБАЛАМИНА И ФТОРАЛКИЛКОБАЛАМИНОВ НА РОСТ КЛЕТОК E. COLI 113/3 И ПЕРВИЧНУЮ КУЛЬТУРУ ЭМБРИОНАЛЬНЫХ ФИБРОБЛАСТОВ ЧЕЛОВЕКА

Онкологический научный центр АМН СССР, Всесоюзный научно-исследовательский витаминный институт, Москва

Исследовано влияние метилкобаламина и его структурного аналога на рост первичной культуры эмбриональной ткани человека. Сравнительный анализ функциональной активности некоторых фторалкилкобаламинов предварительно осуществлен на ауксотрофном по витамину B_{12} и метионину штамме E. coli 113/3. Дифторхлорметилкобаламин (CF_2Cl-Cbl) оказывал наибольшее ингибирующее действие на рост бактериальных клеток в среде с метилкобаламином. Влияние метилкобаламина и дифторхлорметилкобаламина на пролиферативную активность эмбриональных фибробластов человека изучено в средах разного состава. Пролиферативная активность фибробластов в разные сроки культивирования существенно возрастала в среде с метилкобаламином: увеличивались доля меченных ^{3}H-тимидином

› A

ids. V. 2. New York, 1965, p. 622.—
:t.», 1974, v. 117, p. 593—600. —
iochemistry (Wash.)», 1969, v. 8,
iro, Yamamoto Kozo. —
berts J., Holcenberg J. S.,
p. 84. — 6. Holcenberg J. S.,
72, v. 247, p. 7750. — 7. Rama-
. M. — «Arch. Biochem.», 1964, v. 108,
R., Abe T. — «Biochim. biophys.
:. P., Николаев А. Я., Кова-
№ 1, с. 29. — 10. Davis B. J.,
. 321. — 11. Коваленко Н. А.,
охимия», 1970, т. 35, с. 670. —
- «Вопр. мед. химии», 1949, № 1,
нные методы в биохимии. Т. 1. М.,
- «Acta chem. scand.», 1959, v. 13,
ey R. C. — «Biochem. J.», 1967,
ter F. H. — «J. biol. Chem.», 1972,
енко Н. А. — «Успехи биол. хи-
Röhm K. H., Schneider F. —
олов Н. Н., Николаев А. Я.,
40, с. 631.

Поступила 7/VI 1976 г.

INHIBITORS AND KINETICS
MINASE) FROM PSEUDOMONAS
AG

i, A. Ya. Nikolaev

Laboratory of Enzymology, Academy
SSR, Moscow

'seudomonas fluorescens AG was shown
:tively. Besides, the enzyme exhibited
d D-glutamine (70% and 100%, respec-
ides — of glycyl-L-asparagine (40%).
ghtly (5%). Effect of several substrate
ell. Albiciine (α-amino-β-ureide propio-
. β-Methyl aspartic acid, S-carbamoyl
itory effect (20%). Amount of active
[^{14}C-albiciine. Deamidase AG had ap-
onship between the rate of reaction and
vas found to follow Michaelis-Menten

УДК 612.014.3:612.6.015.641.61

узнецова, М. О. Раушенбах,
а, А. М. Юркевич

ТОРАЛКИЛКОБАЛАМИНОВ
13/3 И ПЕРВИЧНУЮ
ИБРОБЛАСТОВ ЧЕЛОВЕКА

зный научно-исследовательский вита-
осква

уктурного аналога на рост первичной
ьный анализ функциональной активно-
льно осуществлен на ауксотрофном
Дифторхлорметилкобаламин (CF$_2$Cl-
ост бактериальных клеток в среде с ме-
порхлорметилкобаламина на пролифе-
еловека изучено в средах разного состав-
ные сроки культивирования существен-
ивались доля меченных ^{3}H-тимидином

клеток и митотический индекс. Достоверное снижение числа клеток, синтезирующих ДНК, и их митотической активности отмечено в среде с дифторхлорметилкобаламином. Результаты исследований на бактериальных клетках и первичной культуре эмбриональных фибробластов человека позволяют охарактеризовать влияние дифторхлорметилкобаламина на процессы клеточной пролиферации как антагониста метилкобаламина.

В наших предыдущих исследованиях показано, что введение в организм здоровых животных метилкобаламина стимулирует пролиферативную активность клеток кроветворной ткани [1]. Увеличение числа клеток, синтезирующих ДНК, и возрастание их митотической активности можно, по-видимому, объяснить индуцирующим действием кобаламинов на метилтрансферазную систему в организме животных. Известно, что контроль взаимодействия соединений фолиевой кислоты и кобаламинов, определяющих интенсивность клеточной пролиферации, осуществляется с помощью такой ферментативной реакции в процессе биосинтеза метионина [2—5]. Экспериментальные исследования подтверждают значительное увеличение активности кобаламинзависимой N^5-метил-ТГФК-гомоцистеин-метилтрансферазы (ЕС 2.1.1.13) в нормальных клетках млекопитающих и опухолевых клетках человека (HeLa, HEp-2), культивируемых в среде, лишенной метионина, но содержащей гомоцистеин и циано- или оксикобаламин [6, 7]. Однако нормальные клетки взрослых животных, а также эмбриональные и опухолевые клетки различны по своей способности расти в среде в отсутствие метионина и образовывать активный холофермент при субоптимальной концентрации витамина B_{12} [8—10]. Поэтому оценка функциональной роли метилкобаламина — кофермента метионинсинтетазной реакции — в процессе роста клеток различных тканей заслуживает особого внимания.

В настоящем сообщении представлены результаты наших исследований влияния метилкобаламина и некоторых его структурных аналогов — фторалкилкобаламинов — на рост бактериальных клеток и первичной культуры эмбриональных фибробластов человека.

Методика

Метилкобаламин и фторалкилкобаламины были получены по известному методу [11], модифицированному в разделе выделения [12]. Сравнительный анализ функциональной активности фторалкилкобаламинов был осуществлен на ауксотрофном штамме по витамину B_{12} и метионину E. coli 113/3 [13]. Состав среды и особенности культивирования бактериального штамма изложены в микробиологическом методе определения витамина B_{12} в тканях [14]. Нами испытано в данной системе 4 соединения: дифторметилкобаламин (CF_2H-Cbl), дифторхлорметилкобаламин (CF_2Cl-Cbl), трифторметилкобаламин (CF_3-Cbl) и перфторпропилкобаламин (C_3F_7-Cbl) [1]. Влияние каждого соединения на рост суточной культуры E. coli 113/3 (125—150 тыс. микробных тел на 1 мл среды) в присутствии оксиили метилкобаламина оценивали после 24 ч инкубации при 37° по изменению оптической плотности среды. Наиболее активное соединение (CF_2Cl—Cbl) было исследовано далее в первичной культуре эмбриональной ткани человека. С этой целью использованы эмбриональные фибробласты человека, культивируемые по известной методике [16]. Выросшую в виде монослоя культуру во флаконах Карелля на 4-е сутки пересевали в количестве 150—200 тыс. клеток на 1 мл в пенициллиновые сосуды с покровными стеклами (2 мл). В различных сериях опытов использовали либо среду 199, либо минимальную среду Игла (без метионина и фолиевой кислоты, содержащей 0,1 мМ DL-гомоцистеина) с добавлением 10% бычьей сыворотки. Исследуемые соединения вносили одновременно с посевом культуры в пенициллиновые флаконы. Растворы кобаламинов в каждом опыте приготовляли непосредственно перед внесением в среду в конечной концентрации $(2{,}5—5{,}0)\cdot10^{-6}$ М (метилкобаламина) и $2{,}5—15{,}0\cdot10^{-3}$ М (дифторхлорметилкобаламина — CF_2Cl—Cbl) с соблюдением условий, препятствующих разрушающему действию света, в течение всего периода культивирования. Оценку роста первичной культуры эмбриональных фибробластов человека в средах разного состава проводили по числу клеток, синтезирующих ДНК, и по их митотической активности. В культуры, предназначенные для авторадиографического анализа, до фиксации вносили меченый ^{3}H-тимидин (удельная активность 11,9 Ки/ммоль) в концентрации 0,5 мкКи/мл на 1—4 ч. При культивировании клеток в минимальной среде

[1] Номенклатура соединений дана в соответствии с рекомендацией комиссии по биохимической номенклатуре [15].

Игла применяли постоянную метку ^{3}H-тимидином (0,1 мкКи/мл) в течение 25, 48, 60 и 72 ч. Фиксацию клеток, растущих на покровных стеклах, осуществляли в смеси спирт — уксусная кислота (3:1) в основном через 24, 48 и 72 ч, а в некоторых случаях — через 64, 69 и 144 ч роста культуры. Препараты подвергали стандартной авторадиографической обработке с использованием эмульсии М [17]. На автограммах, окрашенных азур II-эозином по Романовскому, определяли индекс метки (ИМ) и митотический индекс (МИ) при подсчете не менее 3000 клеток. На каждый срок исследования анализировали 3—4 препарата. Результаты исследований обработаны статистическим методом с оценкой достоверности по критерию *t* Стьюдента.

Результаты и обсуждение

Единственным сообщением относительно биологической активности фторалкилкобаламинов являются исследования Вуда и соавт., показавшие, что некоторые фторпроизводные метилкобаламина (CF_3CH-, CF_2Cl—Cbl) могут ингибировать ферментативное образование метана бесклеточными экстрактами метаногенных бактерий [11]. В наших условиях исследуемые фторалкилкобаламины обнаруживали различную функциональную активность в среде, содержащей окси- или метилкобаламины. Интенсивность роста суточной культуры E. coli 113/3, что характерно для данного штамма, возрастала с увеличением содержания в среде окси- или метилкобаламина от 0,5 до $2{,}0 \cdot 10^{-8}$ М. Трифторметилкобаламин (CF_3—Cbl) и перфторпропилкобаламин (C_3F_7—Cbl) в концентрации $(6—10) \cdot 10^{-3}$ М не подавляли роста клеток этого штамма в присутствии метилкобаламина в среде. Рост культуры был отмечен также в контрольных пробах, в которых среда содержала каждый из указанных аналогов в отсутствие метилкобаламина. Дифторхлор- и дифторметилкобаламины вызывали торможение роста клеток E. coli 113/3 в среде, содержащей метилкобаламин. Наибольшее ингибирующее влияние обнаруживал дифторхлорметилкобаламин. При концентрации в среде $(2—2{,}5) \cdot 10^{-3}$ М CF_2Cl—Cbl и оптимальной концентрации метилкобаламина $(2—2{,}5) \times \times 10^{-8}$ М к 24 ч инкубации отмечалось полное отсутствие роста культуры. Концентрация CF_2Cl—Cbl менее $1{,}0 \times 10^{-3}$ М не вызывала изменения стандартного роста клеток (рис. 1). В присутствии $1{,}0 \cdot 10^{-3}$ М CF_2Cl—Cbl интенсивность роста культуры определялась в зависимости от содержания метилкобаламина в среде. При его минимальной концентрации в среде наблюдалось некоторое замедление роста клеток, однако при увеличении концентрации метилкобаламина до $2{,}0 \times 10^{-8}$ М присутствие $1{,}0 \cdot 10^{-3}$ М дифторхлорметилкобаламина практически уже не влияло на рост культуры (см. рис. 1).

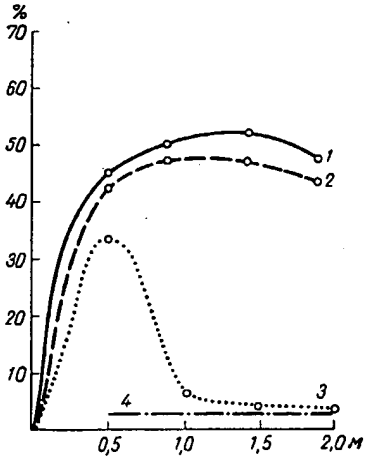


Рис. 1. Рост E. coli 113/3 в солевой среде при различной концентрации дифторхлорметилкобаламина.

По оси абсцисс — концентрация метилкобаламина в среде $(0{,}5—2{,}0) \cdot 10^{-8}$ М; по оси ординат — оптическая плотность среды (в %).
1 — стандартный рост клеток данного штамма в солевой среде с метилкобаламином; *2—4* — то же с возрастающей концентрацией дифторхлорметилкобаламина: *2* — $0{,}25—0{,}5—0{,}75—1{,}0 \cdot 10^{-3}$ М, *3* — $1{,}0—2{,}0—3{,}0—4{,}0 \cdot 10^{-3}$ М, *4* — $2{,}5—5{,}0—7{,}5—10{,}0 \cdot 10^{-3}$ М.

Анализ данных, полученных на специальном бактериальном штамме с кобаламинзависимой метилтрансферазой, в сопоставлении с результатами ранее проведенных исследований [11] позволяет объяснить наиболее эффективное ингибирующее действие дифторхлорметилкобаламина большей устойчивостью Co—C-связи в молекуле этого аналога. Отсутствие торможения роста культуры при воздействии трифторметилкобаламина и перфторпропилкобаламина, по-видимому, зависит от большей лабильности кобальт-углеродной связи, коррелирующей с электроотрицательностью

0,1 мкКи/мл) в течение 25, 48, 60 и
клах, осуществляли в смеси спирт —
ı, а в некоторых случаях — через 64,
ндартной авторадиографической обра-
ммах, окрашенных азур II-эозином по
готический индекс (МИ) при подсчете
я анализировали 3—4 препарата. Ре-
етодом с оценкой достоверности по

суждение

ьно биологической активности
вания Вуда и соавт., показав-
метилкобаламина (CF_3CH-,
ивное образование метана бес-
терий [11]. В наших условиях
е фторалкилкобаламины обна-
различную функциональную ак-
среде, содержащей окси- или
амины. Интенсивность роста су-
ьтуры E. coli 113/3, что харак-
данного штамма, возрастала с
м содержания в среде окси- или
амина от 0,5 до $2{,}0\cdot10^{-8}$ М.
тилкобаламин (CF_3—Cbl) и пер-
лкобаламин (C_3F_7—Cbl) в кон-
$(6—10)\cdot10^{-3}$ М не подавляли
ок этого штамма в присутствии
амина в среде. Рост культуры
н также в контрольных пробах,
среда содержала каждый из
аналогов в отсутствие метил-
а. Дифторхлор- и дифторметил-
ы вызывали торможение роста
coli 113/3 в среде, содержащей
амин. Наибольшее ингибирую-
ие обнаруживал дифторхлорме-
ин. При концентрации в среде
$^{-3}$ М CF_2Cl—Cbl и оптимальной
ции метилкобаламина (2—2,5)×
к 24 ч инкубации отмечалось
утствие роста культуры. Кон-
CF_2Cl—Cbl менее $1{,}0\times10^{-3}$ М
а изменения стандартного роста
с. 1). В присутствии $1{,}0\cdot10^{-3}$ М
определялась в зависимости от
его минимальной концентрации
роста клеток, однако при уве-
до $2{,}0\times10^{-8}$ М присутствие
практически уже не влияло на

иальном бактериальном штамме
в сопоставлении с результатами
зволяет объяснить наиболее эф-
рхлорметилкобаламина большей
гого аналога. Отсутствие тормо-
трифторметилкобаламина и пер-
висит от большей лабильности
цей с электроотрицательностью

верхнего аксиального лиганда. В наших дальнейших исследованиях в связи с этим мы использовали дифторхлорметилкобаламин, способный ингибировать в бактериальных клетках не только метанообразование [11], но и активность кобаламинзависимой метилтрансферазы в процессе биосинтеза метионина.

Сравнительное изучение пролиферации первичной культуры эмбриональных фибробластов человека непосредственно после посева на среды разного состава позволило оценить влияние кобаламинов уже в ранний период адаптации клеток к новым условиям культивирования.

Таблица 1

Изменение числа клеток, синтезирующих ДНК, первичной культуры эмбриональных фибробластов человека в среде 199, содержащей кобаламины

Добавление к основной среде	Концентрация кобаламинов, моль	Возраст культуры к моменту фиксации, ч	Индекс меченых клеток, %	Отношение индекса метки в опытной и контрольной культурах
Среда 199 (контроль)	—	48	18,5±0,4	—
		72	16,6±0,4	
Метилкобаламин	$2{,}5\cdot10^{-6}$	48	28,3±0,9	1,4
		72	20,6±0,8	1,2
	$5{,}0\cdot10^{-6}$	48	25,1±1,2	1,2
		72	16,8±0,5	1,0
	$2{,}5\cdot10^{-3}$	48	17,5±0,9	1,0
		72	12,3±0,8	0,77
CF_2Cl—Cbl	$5{,}0\cdot10^{-3}$	48	15,2±0,7	0,88
		72	12,4±0,9	0,75

Примечание. Приведены средние величины индекса меченых клеток по результатам 3—4 опытов; уровень значимости различий $P<0{,}01$.

Анализ пролиферативной активности фибробластов в среде 199, содержащей метилкобаламин, на 2—3-и сутки роста культуры обнаружил существенное увеличение числа клеток, синтезирующих ДНК. В среде с метилкобаламином индекс меченных ^{3}H-тимидином клеток по средним результатам опытов превышал контроль в 1,4 раза к 48 ч культивирования (табл. 1). При наиболее оптимальной концентрации $2{,}5\cdot10^{-6}$ М метилкобаламина существенно больше клеток (28,3 и 20,6%; $P<0{,}001$) отмечалось к 48 и 72 ч культивирования в фазе синтеза ДНК. С увеличением в среде концентрации метилкобаламина до $5{,}0\cdot10^{-6}$ М не наблюдалось дальнейшего возрастания индекса меченых клеток. Стимулирующий эффект метилкобаламина в максимальной дозе постоянно был несколько менее выражен, и к 72 ч роста культуры число клеток, синтезирующих ДНК, практически снижалось до уровня контрольных образцов (16,8%).

Величина митотического индекса фибробластов незначительно колебалась в отдельных опытах, но в среднем число делящихся клеток в прослеженные сроки от 24 до 144 ч роста культуры не превышало 22%$_0$. В среде 199 митотическая активность эмбриональных фибробластов достигала своего максимального значения на 2-е сутки роста (10%$_0$), постепенно снижаясь после 72 ч культивирования. В среде 199, содержащей метилкобаламин, митотическая активность первичной культуры эмбриональных фибробластов человека была выше. Максимальная величина митотического индекса в этих условиях (20%$_0$) была отмечена также к 48 ч культивирования. При этом митотическая активность фибробластов определялась в прямой зависимости от концентрации метилкобаламина в среде. Число делящихся клеток при концентрации $2{,}5\cdot10^{-6}$ и $5{,}0\cdot10^{-6}$ М метилкобаламина была соответственно в 1,5—2 раза выше, чем в контрольной культуре (рис. 2). Нами исследовано также влияние дифторхлорметилкобаламина на рост первичной культуры эмбриональных фибробластов человека в среде 199.

В этих условиях культивирования значительно меньше фибробластов находилось в фазе синтеза ДНК на 2—3-и сутки культивирования, чем в контрольных культурах и в среде с метилкобаламином. Наиболее отчетливый эффект снижения числа клеток, синтезирующих ДНК, был выявлен в 48- и 72-часовой культуре в среде 199, содержащей $5{,}0 \cdot 10^{-3}$ М CF_2Cl—Cbl (см. табл. 1). При уменьшении его концентрации в среде до $2{,}5 \cdot 10^{-3}$ М достоверное снижение числа клеток, меченных ^{3}H-тимидином, наблюдалось к 72 ч культивирования.

Митотическая активность фибробластов в среде 199 с различной концентрацией CF_2Cl—Cbl существенно не изменялась. Митотический индекс клеток, выращиваемых при концентрации $2{,}5 \cdot 10^{-3}$ М CF_2Cl—Cbl, к 48—

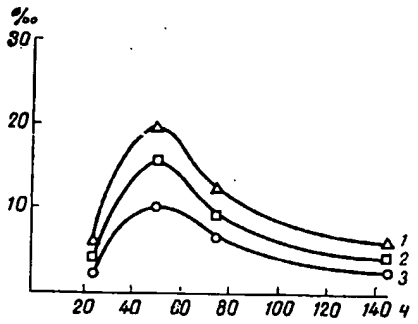


Рис. 2. Возрастание митотической активности эмбриональных фибробластов человека в среде 199 с метилкобаламином.

По оси абсцисс — срок культивирования (в ч); по оси ординат — митотический индекс (в ‰). Каждая точка представляет среднюю величину результатов 4 опытов. Среда 199 с 10% бычьей сыворотки (контроль *3*), с добавлением метилкобаламина в концентрации $2{,}5 \cdot 10^{-6}$ М (*2*), $5{,}0 \cdot 10^{-6}$ М (*1*).

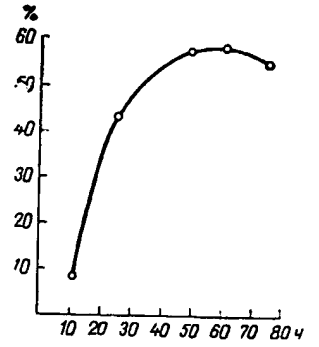


Рис. 3. Динамика включения ^{3}H-тимидина в эмбриональные фибробласты человека в разные сроки культивирования (минимальная среда Игла с 10% недиализированной бычьей сыворотки).

По оси абсцисс — время инкубации клеток с ^{3}H-тимидином и возраст культуры после смены среды (в ч); по оси ординат — число меченых клеток (в %).

72 ч практически не отличался от контроля. Однако при увеличении концентрации дифторхлорметилкобаламина в среде 199 до $5{,}0 \cdot 10^{-3}$ М митотическая активность фибробластов на 2-е сутки роста была ниже, чем в контроле, хотя выявляемое различие не было существенным ($9{,}0 \pm 2{,}03$ и $12{,}0 \pm 0{,}7\%_0$ соответственно по средним результатам 7 опытов).

Учитывая возможность специфического действия данного соединения как функционального антагониста метилкобаламина, в дальнейшем мы исследовали влияние более высокой концентрации дифторхлорметилкобаламина на рост эмбриональных фибробластов человека в среде Игла, лишенной фолиевой кислоты и метионина. В наших условиях рост клеток был лимитирован минимальным содержанием метионина, фолиевой кислоты (преимущественно 5-метил-ТГФК) и кобаламинов, которое обеспечивалось добавлением к среде 10% недиализированной бычьей сыворотки. Присутствие в сыворотке транскобаламина II являлось также необходимым фактором для поступления кобаламинов в клетки.

Характер пролиферации эмбриональных фибробластов, выращиваемых на среде Игла, имел те же общие закономерности, которые были выявлены другими авторами при исследовании первичных культур эмбриональных фибробластов человека и мыши [18, 19]. Примерно через 20 ч после пересева культуры наблюдалось постепенное увеличение интенсивности пролиферации клеток, которое достигало максимального значения на 2-е сутки. В наших опытах при постоянной инкубации клеток с ^{3}H-тимидином число меченых клеток через 6 ч после пересева культуры составля-

ьно меньше фибробластов на-
утки культивирования, чем в
баламином. Наиболее отчетли-
ирующих ДНК, был выявлен
ржащей $5{,}0 \cdot 10^{-3}$ М CF_2Cl—Cbl
ации в среде до $2{,}5 \cdot 10^{-3}$ М до-
х ^{3}Н-тимидином, наблюдалось

в среде 199 с различной кон-
енялась. Митотический индекс
$2{,}5 \cdot 10^{-3}$ М CF_2Cl—Cbl, к 48—

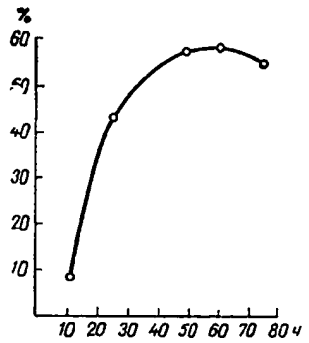


Рис. 3. Динамика включения ^{3}Н-тимидина в эмбриональные фибробласты человека в разные сроки культивирования (минимальная среда Игла с 10% недиализированной бычьей сыворотки).

По оси абсцисс — время инкубации клеток с ^{3}Н-тимидином и возраст культуры после смены среды (в ч); по оси ординат — число меченых клеток (в %).

. Однако при увеличении кон-
среде 199 до $5{,}0 \cdot 10^{-3}$ М мито-
сутки роста была ниже, чем в
ыло существенным ($9{,}0 \pm 2{,}03$ и
зультатам 7 опытов).
действия данного соединения
обаламина, в дальнейшем мы
трации дифторхлорметилкобал-
человека в среде Игла, лишен-
их условиях рост клеток был
метионина, фолиевой кислоты
минов, которое обеспечивалось
й бычьей сыворотки. Присутст-
ось также необходимым факто-
ки.
ых фибробластов, выращивае-
ономерности, которые были вы-
ли первичных культур эмбрио-
[18, 19]. Примерно через 20 ч
тепенное увеличение интенсив-
игало максимального значения
ной инкубации клеток с ^{3}Н-ти-
сле пересева культуры составля-

ло всего 10% (рис. 3). В дальнейшем индекс меченых клеток постепенно нарастал и к 48 ч достигал максимальной величины (59%), сохраняясь на этом уровне и в 72-часовой культуре. Таким образом, период наиболее активной пролиферации клеток первичной культуры эмбриональных фибробластов человека продолжался около 2 сут. При этом пул пролиферирующих клеток в 2—3-суточной культуре составлял около 60%, т. е. довольно большая часть клеток культуры (около 40%) находилась вне митотического цикла.

Таблица 2

Изменение пролиферативной активности эмбриональных фибробластов человека в минимальной среде Игла при добавлении кобаламинов

Добавление к основной среде	Концентрация кобаламинов, моль	Возраст культуры к моменту фиксации, ч	Митотический индекс, ‰	Индекс меченых клеток, %	Соотношение митотических индексов в опытной и контрольной культурах	Соотношение индексов меченых клеток в опытной к контрольной культурах
Среда Игла (контроль)		48	10,50±2,10	11,90±1,10		
		64		11,53±1,20	—	—
		69		7,98±1,20		
		72	6,30±1,10	13,30±0,75		
Метилкобаламин	$2{,}5 \cdot 10^{-6}$	64		21,30±0,70		1,8
		69		14,80±1,80	—	
Метилкобаламин и цианокобаламин	$2{,}5 \cdot 10^{-6}$	64		25,90±1,10		2,2*
		69		17,20±0,78		2,2
	$1{,}0 \cdot 10^{-2}$	72	18,16±1,07		2,8	
CF_2Cl—Cbl	$1{,}0 \cdot 10^{-2}$	48	10,75±4,00	12,50±0,75	1,0	
		72	3,83±0,86	8,00±0,72	0,61	0,60
	$1{,}5 \cdot 10^{-2}$	48	7,90±4,40*	13,50±0,57	0,75	
		72	3,00±0,71	9,43±0,55	0,48	0,70

Примечание. Звездочкой отмечен уровень значимости различий $P<0{,}05$, в остальных опытах $P<0{,}001$.

Рост клеток в минимальной среде Игла, содержащей метилкобаламин, существенно отличался от контрольной культуры. В среде Игла с метилкобаламином к 48 и 72 ч культивирования значительно больше клеток включало ^{3}Н-тимидин (табл. 2). Максимальное число клеток, синтезирующих ДНК, было выявлено в среде, содержащей смесь цианo- и метилкобаламинов. Индекс меченых фибробластов к 64—69 ч культивирования при импульсной метке с ^{3}Н-тимидином более чем в 2 раза превышал контроль (см. табл. 2). Наибольший эффект их комбинированного действия является, по-видимому, результатом дополнительного образования в клетках коферментных форм витамина B_{12}, как отмечалось на фибробластах кожи человека [20]. Полученные данные показывают, что при добавлении метилкобаламина к основной среде (199 или Игла) более интенсивно осуществляется не только период адаптации клеток к новым условиям, но и активируется их вступление в фазу синтеза ДНК. Действительно, при пересеве первичной культуры кожи эмбрионов овцы в среду с цианокобаламином значительно больше клеток к 48—72 ч прикрепляется к стеклу и выходит из фазы торможения [21]. Известно, что время проникновения в клетку цианокобаламина измеряется минутами, однако для процесса биосинтеза коферментов витамина B_{12} и образования холофермента необходимо около 18 ч [22]. Очевидно, в присутствии метилкобаламина в клетках индуцируется активность метилтрансферазы аналогично воздействию оксикобаламина в культуре клеток почек новорожденных хомячков.

В сходных условиях культивирования активность 5-метил-ТГФК-гомоцистеин-метилтрансферазы в клетках в присутствии оксикобаламина достигала максимального значения к 24 ч и оставалась стабильной не менее 3 сут [7]. В наших исследованиях при культивировании эмбриональных фибробластов человека в среде с метилкобаламином существенно больше клеток к 48—72 ч находилось в фазе синтеза ДНК и их митотическая активность значительно превышала контроль (см. табл. 2). Данные о стимулирующем влиянии метилкобаламина на функциональное состояние эмбриональных фибробластов согласуются с результатами недавних исследований о снижении активности 5-метил-ТГФК-гомоцистеин-метилтрансферазы в фибробластах человека при дефиците метилкобаламина [23]. В противоположность стимулирующему влиянию метилкобаламина присутствие в среде его структурного аналога затрудняло этот этап, задерживая вступление клеток в фазу синтеза ДНК и их последующее деление. В минимальной среде Игла с высокой концентрацией $CF_2Cl—CbI$ $(1{,}0—1{,}5)\cdot10^{-2}$ М) пролиферативная активность фибробластов была существенно ниже к 72 ч культивирования. Доля меченых клеток и величина митотического индекса при этом были в 1,5—1,7 раза ниже, чем в контрольной культуре, особенно по сравнению с интенсивностью роста клеток в среде, содержащей метилкобаламин (см. табл. 2). Можно полагать, что в среде с дифторхлорметилкобаламином при дефиците метионина и фолиевой кислоты тормозится метилирование гомоцистеина, а вследствие этого образование метионина и ТГФК, ограничивая тем самым в клетках процесс биосинтеза ДНК.

Совокупность результатов исследований, проведенных на бактериальных клетках и в культуре эмбриональных фибробластов человека, позволяет, таким образом, охарактеризовать влияние дифторхлорметилкобаламина как функционального антагониста метилкобаламина. Полученные данные определяют необходимость изучения механизма действия антагонистов метилкобаламина в процессах пролиферации клеток in vivo. Эти вопросы составляют предмет наших исследований в настоящее время.

ЛИТЕРАТУРА

1. Голенко О. Д., Мясищева Н. В., Раушенбах М. О. и др. — «Вопр. мед. химии», 1974, № 5, с. 549. — 2. Burke G. T., Mangum J. H. Brodie J. D. et a. — «Biochemistry (Wash.)», 1970, v. 9, p. 4297. — 3. Мясищева Н. В. В кн.: Роль эндогенных факторов в развитии лейкозов. М., 1974, с. 111. — 4. Taylor R. T., Hanna M. L. — «Arch. Biochem.», 1974, v. 165, p. 787. — 5. Weyden M. B. van der, Cooper M., Firkin B. G. — «Blood», 1973, v. 41, p. 299. — 6. Mangum J., Murray B. K., Morth J. — «Biochemistry (Wash.)», 1969, v. 8, p. 3496. — 7. Kamely D., Littlefild J. W., Erbe R. W. — «Proc. nat. Acad. Sci. USA», 1973, v. 70, p. 2585. — 8. Mudd S. H., Levy H. L., Abeles R. H. — «Biochem. biophys. Res. Commun.», 1969, v. 35, p. 121. — 9. Ashe H., Clark B. R., Chu F. et a. — Ibid., 1974, v. 57, p. 417. — 10. Helpern B. C., Clark B. R., Hardy D. N., et a. — «Proc. nat. Acad. Sci. USA», 1974, v. 71, p. 1133. — 11. Penley M. W., Brown D. G., Wood J. M. — «Biochemistry (Wash.)», 1970, v. 9, p. 4302. — 12. Тачкова Е. М., Рудакова И. П., Мясищева Н. В. и др. — «Биоорган. химия», 1976, № 2, с. 535. — 13. Taylor R. T., Dickerman H., Weissbach A. — «Arch. Biochem.». 1966, v. 117, p. 405. — 14. Букин В. Н., Арешкина Л. Я., Куцева Л. С. — «Биохимия», 1954, т. 19, с. 43. — 15. «Biochemistry (Wash.)», 1974, v. 13, p. 1550. — 16. Кузнецова Л. Е. — «Бюлл. экспер. биол.», 1969, № 2, с. 107. — 17. Епифанова О. И., Терских В. В. Метод радиоавтографии в изучении клеточных циклов. М., 1969. — 18. Зосимовская А. И., Ляпунова Н. А. — «Цитология», 1966, № 8, с. 208. — 19. Бродский В. Я., Васильев Ю. М., Гельфанд И. М. и др. — «Цитология», 1974, т. 16, с. 1369. — 20. Mahoney M. J., Rosenberg L. E. — «J. Lab. clin. Med.», 1971, v. 78, p. 302. — 21. Новикова Н. П., Штиккель Э. И., Голубчикова В. С. и др. — «Цитология», 1973, т. 15, с. 351. — 22. Peirce K., Abe T., Cooper B. A. — «Biochim. biophys. Acta», 1975, v. 381, p. 348. — 23. Dillon M. J., England J. M., Gompertz D. et a. — «Clin. Sci.», 1974, v. 47, p. 43.

Поступила 19/VI 1976 г.

ктивность 5-метил-ТГФК-гомо-
исутствии оксикобаламина до-
ставалась стабильной не менее
льтивировании эмбриональных
баламином существенно боль-
интеза ДНК и их митотическая
ль (см. табл. 2). Данные о сти-
на функциональное состояние
я с результатами недавних ис-
етил-ТГФК-гомоцистеин-метил-
ефиците метилкобаламина [23].
лиянию метилкобаламина при-
затрудняло этот этап, задержи-
К и их последующее деление.
ой концентрацией CF_2Cl—Cbl
вность фибробластов была су-
оля меченых клеток и величина
,5—1,7 раза ниже, чем в конт-
с интенсивностью роста клеток
табл. 2). Можно полагать, что
и дефиците метионина и фолие-
моцистеина, а вследствие этого
ая тем самым в клетках процесс

ий, проведенных на бактериаль-
фибробластов человека, позво-
лияние дифторхлорметилкобал-
метилкобаламина. Полученные
ия механизма действия антаго-
лиферации клеток in vivo. Эти
ледований в настоящее время.

РА

., Раушенбах М. О. и др. —
rke G. T., Mangum J. H. Bro-
), p. 4297. — 3. Мясищева Н. В.
йкозов. М., 1974, с. 111. — 4. Tay-
», 1974, v. 165, p. 787. — 5. Wey-
. G. — «Blood», 1973, v. 41, p. 299. —
— «Biochemistry (Wash.)», 1969, v. 8,
N., Erbe R. W. — «Proc. nat. Acad.
H., Levy H. L., Abeles R. H. —
. 121. — 9. Ashe H., Clark B. R.,
10. Helpern B. C., Clark B. R.,
SA», 1974, v. 71, p. 1133. — 11. Pen-
«Biochemistry (Wash.)», 1970, v. 9,
. И. П., Мясищева Н. В. и др. —
Taylor R. T., Dickerman H.,
v. 117, p. 405. — 14. Букин В. Н.,
имия», 1954, т. 19, с. 43. — 15. «Bio-
узнецова Л. Е. — «Бюлл. экспер.
а О. И., Терских В. В. Метод ра-
1969. — 18. Зосимовская А. И.,
3, с. 208. — 19. Бродский В. Я.,
— «Цитология», 1974, т. 16, с. 1369. —
J. Lab. clin. Med.», 1971, v. 78, p. 302. —
Голубчикова В. С. и др. — «Ци-
., Abe T., Cooper B. A. — «Bio-
3. Dillon M. J., England J. M.,
17, p. 43.

Поступила 19/VI 1976 г.

EFFECT OF METHYLCOBALAMINE AND FLUOROALKYLCOBALAMINES ON GROWTH OF E. COLI 113/3 CELLS AND ON THE PRIMARY CULTURE OF HUMAN EMBRYONAL FIBROBLASTS

N. V. Myasischeva, O. D. Golenko, L. E. Kuznetsova, M. O. Raushenbakh, I. P. Rudakova, E. M. Tachkova, A. M. Yurkevich

Research Centre of Oncology, Academy of Medical Sciences of the USSR, All-Union Research Vitamin Institute, Moscow

Comparative analysis of the functional activity of several fluoroalkylcobalamines was carried out using E. coli 113/3 strain deficient in vitamin B_{12} and methionine. Difluoro chlor methylcobalamine (CF_2Cl-Cbl) exhibited the most distinct inhibitory effect on growth of bacterial cells in the medium with cobalamine. Effect of methylcobalamine and CF_2Cl-Cbl on the proliferative activity of human embryonal fibroblasts was studied in media of various composition. The proliferative activity of fibroblasts was distinctly increased in the medium with methylcobalamine at various periods of cultivation; the fraction of ^3H-thimidine labelled cells and the mitotic index were increased. The distinct decrease in amount of cells, synthesizing DNA, and in their mitotic activity was observed in medium with CF_2Cl-Cbl. The data obtained suggest that difluorochlor methylcobalamine affects the cell proliferation as the antagonist of methylcobalamine in experiments with bacterial cells and the primary culture of human embryonal fibroblasts.

УДК 612.173.1.015.1:577.152.361

А. Т. Иващенко

ИССЛЕДОВАНИЕ АНИОНЧУВСТВИТЕЛЬНОЙ АТФ-АЗЫ ЯДЕР СЕРДЦА КРЫСЫ

Институт экспериментальной биологии АН Казахской ССР, Алма-Ата

Исследованы свойства HCO_3^--стимулируемой АТФ-азы ядер клеток сердца крысы. Максимальная активность HCO_3^--АТФ-азы наблюдается при концентрации бикарбоната 25 мМ. Оптимум pH 8,0—8,5. Бикарбонат стимулирует АТФ-азу только в присутствии Mg^{2+}, Mn^{2+} и Zn^{2+}. С Co^{2+}, Ni^{2+}, Ca^{2+} и Ca^{2+} активации не наблюдается. Na_2CO_3 и Na_2SO_3 в концентрации 30 мМ стимулируют АТФ-азную активность ядер на 20 и 81% соответственно. Анионы N_3^-, I^-, SCN^- и ClO_4^- ингибируют Mg^{2+}-АТФ-азу и HCO_3^--АТФ-азу. Ионы SO_4^{2-} и HSO_3^- не влияют на АТФ-азную активность ядер.

HCO_3^--стимулируемая АТФ-аза обнаружена в ядерной фракции клеток слизистой оболочки желудка [1, 2], головного мозга [3], почек [4], печени [5] и опухолей [6]. В ядрах мышечной ткани свойства аниончувствительной АТФ-азы не изучены. Интерес к исследованию свойств бикарбонат-активируемой АТФ-азы обусловлен также неопределенностью ее физиологической функции. В настоящей работе изучали свойства HCO_3^--стимулируемой АТФ-азы клеток сердца крысы с целью сравнения их со свойствами HCO_3^--АТФ-аз различных тканей.

Методика

Опыты проводили на белых беспородных крысах массой 150—200 г. После декапитации животных сердце сразу извлекали и измельчали на холоду в среде 1 : 0,25 М сахароза, 10 мМ трис-HCl-буфер pH 7,4. Измельченную ткань продавливали через сетку с диаметром пор 0,6 мм и гомогенизировали в среде 1. Гомогенат фильтровали через 4 слоя марли. Ядра получали по модифицированной методике [7]. Фильтрат центрифугировали 2 мин при 100 g с целью осаждения крупных клеточных фрагментов. Осадок отбрасывали, а надосадочную жидкость центрифугировали 10 мин при 900 g. Полученный осадок, содержащий ядра, отмывали 1 раз в среде гомогенизации и ресуспендировали в 5 мл среды 2 : 50 мМ трис-HCl-буфер pH 7,4, 80 мМ KCl и 60 мМ NaCl. Суспензию наслаивали на 10 мл 0,6 М сахарозы и центрифугировали 10 мин при 1200 g. Осевшие на дно ядра отмывали в среде 2. Чистоту препарата контролировали электронно-микроскопическим методом. Митохондрии практически отсутствовали в препаратах. Фракцию митохондрий сердца крысы получали по описанной методике [8] дополнительным однократным отмыванием в среде 2.

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name: Patricia Duffy | Org or A.U.: 1645 | Phone: 305-7555

Serial Number: 05/654,116 | Req. Date: ASAP | Date:

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Hogenkamp et al (authors)/(Eds) Banerjee R.
Journal/Book Title:	Chemistry & Biochemistry of B12
Article Title:	
Volume (Issue):	
Pages:	p. 385-410
Year of Publication:	1999.
Publisher:	John Wiley & Sons - NY
Remarks:	

BOOK

11/05/30

STIC Use Only Accession Number: 397,761

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date			5/31											
Initials			RC											
Results														
Examiner Called														
Page Count			15											
Money Spent			$ 1.35											

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

CHEMISTRY AND BIOCHEMISTRY OF B_{12}

Edited by
RUMA BANERJEE
Department of Biochemistry
University of Nebraska
Lincoln, NE

A Wiley-Interscience Publication
JOHN WILEY & SONS, INC.
New York • Chichester • Weinheim • Brisbane • Singapore • Toronto

D. L., and Francomano, C. A. (1988), *J. Pediatr.* **112**, 32–39.

114. Acques, P. F., Bostom, A. G., Williams, R. R., Ellison, R. C., Eckfeldt, J. H., Rosenberg, I. H., Selhub, J., and Rozen, R. (1996), *Circulation* **93**, 7–9.
115. Kluijtmans, L. A. J., van den Heuvel, L. P. W. J., Boers, G. H. J., Frosst, P., Stevens, E. M. B., van Oost, B. A., den Heijer, M., Trijbels, F. J. M., Rozen, R., and Blom, H. J. (1996), *Amer. J. Hum. Genet.* **58**, 35–41.
116. Frosst, P., Blom, H. J., Milos, R., Goyette, P., Sheppard, C. A., Matthews, R. G., Beoers, G. J. H., den Heijer, M., Kluijtmans, L. A. J., van den Heuvel, L. P., and Rozen, R. (1995), *Nat. Genet.* **10**, 111–113.
117. Van der Put, N. M. J., Steegers-Theunissen, R. P. M., Frosst, P., Trijbels, F. J. M., Eskes, T. K. A. B., van den Heuvel, L. P., Mariman, E. C. M., den Heyer, M., Rozen, R., and Blom, H. J. (1995), *Lancet* **346**, 1070–1071.
118. Christensen, B., Frosst, P., Lussier-Cacan, S., Selhub, J., Goyette, P., Rosenblatt, D. S., Genest, J. J., and Rozen, R. (1997), *Arterioscler. Thromb. Vasc. Biol.* **17**, 569–573.
119. Van der Put, N. M. J., Gabreels, F., Stevens, E. M. B., Smeitink, J. A. M., Trijbels, F. J. M., Eskes, T. K. A. B., van der Heuvel, L. P., and Blom, H. J. (1998), *Amer. J. Hum. Genet.* **62**, 1044–1051.

15

DIAGNOSTIC AND THERAPEUTIC ANALOGUES OF COBALAMIN

HARRY P. C. HOGENKAMP
Department of Biochemistry
University of Minnesota
Minneapolis, MN 55455

DOUGLAS A. COLLINS
Department of Diagnostic Radiology
Mayo Clinic
Rochester, MN 55905

CHARLES B. GRISSOM
Department of Chemistry
University of Utah
Salt Lake City, UT 84112-0850

FREDERICK G. WEST
Department of Chemistry
University of Utah
Salt Lake City, UT 84112-0850

Chemistry and Biochemistry of B_{12}. Edited by Ruma Banerjee.
ISBN 0-471-25390-1.

1 INTRODUCTION

Two enzymes requiring coenzyme forms of vitamin B_{12} (cobalamin) are found in humans. Adenosylcobalamin is the coenzyme for methylmalonyl–CoA mutase (EC 5.4.99.2), a mitochondrial enzyme that catalyzes the interconversion of methylmalonyl CoA and succinyl CoA. Methylcobalamin serves as the coenzyme for ^{5}N-methyltetrahydrofolate–homocysteine methyltransferase (methionine synthase, EC 2.1.1.13). This cytosolic enzyme catalyzes the methyl transfer from ^{5}N-methyltetrahydrofolate to homocysteine with generation of tetrahydrofolate (THF) and methionine. Consequently, methylmalonic acidosis and homocysteinuria frequently occur in patients deficient in the vitamin or patients who suffer from an inherited disorder of cobalamin metabolism.

Due to its production of THF, methionine synthase is indirectly involved in the synthesis of dTMP and thus DNA. In cobalamin deficiency the folates are "trapped" as methyltetrahydrofolate, which limits the availability of THF and the conversion of dUMP to dTMP by thymidylate synthase. As a result, the synthesis of DNA and cell proliferation are impaired.

Several years ago, Hall (1) showed that the transcobalamin II-mediated uptake of ^{57}Co cyanocobalamin by peripheral blood lymphocytes (PBL) was greatly enhanced when the cells were stimulated with a mitogen. He further demonstrated that the increased uptake of cobalamin corresponded to simultaneous increases in methionine synthase activity and DNA synthesis. Hydroxyurea inhibited not only DNA synthesis, but also the transcobalamin II-mediated uptake of ^{57}Co cyanocobalamin.

It has been well documented that rapidly proliferating tissues have a very high uptake of cobalamin. For instance, Flodh (2) found that after intravenous injection of radiolabeled cyanocobalamin in mice, tissues such as the ovarian follicles, testicular tubuli, gastrointestinal mucosa, and fetal tissues, as well as several tumors, including spontaneous mammary carcinomas, Ehrlich ascites tumors, and soft-tissue sarcomas, showed significant accumulation of radioactivity. Especially high levels were observed in rapidly growing soft fibroblastic osteosarcoma tissue.

2 TRANSPORT OF COBALAMINS

Three distinct transport proteins are involved in the absorption and transport of cobalamins: intrinsic factor (IF), transcobalamin II (TC-II), and transcobalamin I (TC-I; also called haptocorrin, HC, and R-protein). (3,4,5). The oxyntic mucosa of the body and fundus of the stomach excrete IF, which is the most specific of the cobalamin-binding proteins. Cyanocobalamin, HOCbl, MeCbl, and AdoCbl bind to intrinsic factor with similar affinities, thereby suggesting that the upper β-axial ligand of cobalt does not influence the binding significantly. On the other hand, modifications of the corrin ring or of the lower 5,6-dimethlbenzimidazole ligand significantly decrease the affinity for IF.

Human TC II is a 43-kDa plasma protein. Both IF and TC II deficiencies lead to abnormalities such as megaloblastic anemia and a demyelinating disorder of the nervous system. In contrast, a deficiency of HC does not produce any detectable clinical abnormalities. Transcobalamin I is a glycoprotein of 33–40% carbohydrate content with 16–19 sialic acid residues. In plasma, TC I turns over very slowly ($t_{½}$ = 10 days) and appears to serve as the major storage protein for cobalamin. Allen (3) has suggested that TC I may participate in the storage of excess cobalamin and bind degraded cobalamin for removal. TC I may also stabilize serum cobalamin against transdermal photolysis.

3 ABSORPTION OF COBALAMINS

Within the proximal small intestine, HC is degraded by pancreatic enzymes, freeing cobalamin to combine with IF. In contrast to HC, IF is highly specific for cobalamin, and the IF–cobalamin complex is resistant to proteolytic digestion. Once this complex reaches the distal small bowel, it interacts with IF-receptor sites on the outer surface of the microvillous membrane in the distal ileum. The process requires the presence of divalent cations, including Ca^{++}. Following uptake into the microvilli, IF is degraded by proteolysis. After transcytosis through the enterocytes, the cobalamin appears in the portal circulation as the TC II–cobalamin (TC II–Cbl) complex, which must then pass through the liver. The cellular uptake of cobalamins bound to TC II occurs in all cells by receptor-mediated endocytosis. After binding to surface receptors. the TC II–Cbl complex is internalized by absorptive endocytosis in clathrin-coated pits and vesicles (7–10). Inside the cell, TC II is degraded or recycled to the cell surface. The cobalamin released intracellularly is reduced to cob(II)alamin. Jacobsen et al. (11) found that in K562 cells, two-thirds of the internalized TC II was degraded, but 30% returned intact to the cell surface and was exocytosed. In the cytosol, cob(II)alamin binds to apomethionine synthase, while in the mitochondria it is further reduced to cob(I)alamin, which reacts with ATP to form AdoCbl, the coenzyme for methylmalonyl CoA mutase. Antibodies to TC II inhibit the transport of cobalamins and block the proliferation of leukemic cells *in vitro* (12).

4 MODIFICATION OF COBALAMINS

Cyanocobalamin (Figure 1) contains several functional groups that can be readily modified. The most obvious and simplest modification is alkylation of the cobalt atom. Reaction of cob(I)alamin with alkylating agents produces a carbon–cobalt (Co–C) bond that is sensitive to light, so that the synthesis and handling of modified cobalamin must be carried out in dim light.

A second possible modification site is the lower 5,6-dimethylbenzimidazole nucleotide. Two free hydroxyl groups (2′ and 5′) are available at the ribofuranose moiety. The 5′-hydroxyl of CNCbl has been esterified with succinic anhydride by Toraya et al. (13) and, more recently, Pathare and coworkers (14), to give the cyanocobalamin–succinate derivative. Takaheta and coworkers (15) prepared hydrophobic derivatives of CNCbl by reacting the vitamin with long-chain fatty acid anhydrides. They suggest that the fatty acids are attached to the 5′-hydroxyl group via an ester linkage.

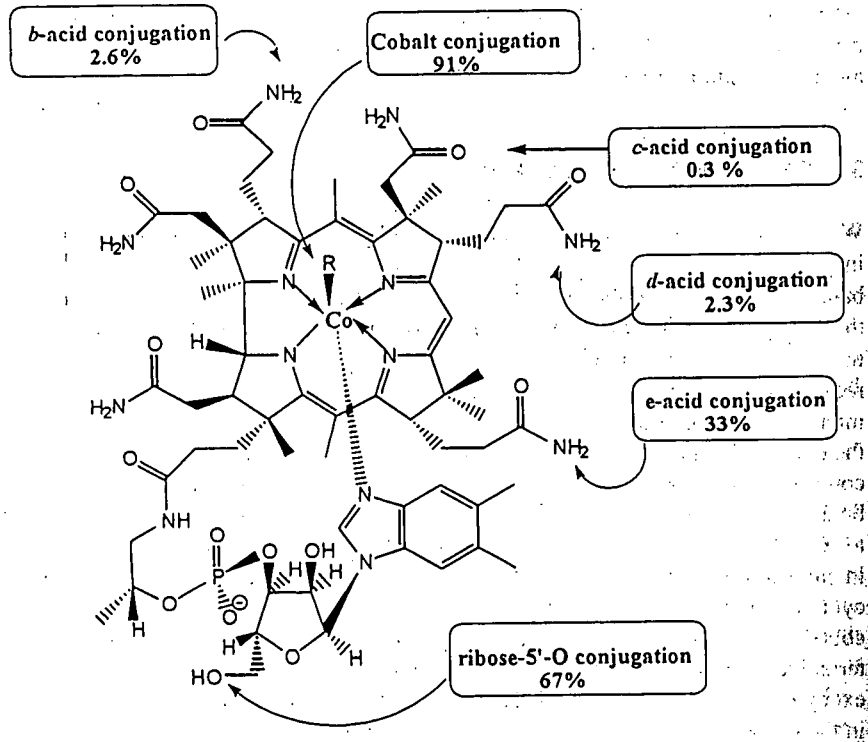


FIGURE 1 Possible modification sites of cobalamin. The percentage indicates the relative binding inhibition versus CN–Cbl in a competitive assay (14). The attachment of ligands at positions other than the cobalt atom significantly decreases recognition by transcobalamin. Data are from Pathare et al. (14).

Poly-L-lysine was coupled to the phosphate moiety in the presence of a water soluble carbodiimide by Olesen et al. (16). Modification of the c-acetamide and of the *b*, *d*, and *e*-propionamides requires lactone formation or acid hydrolysis, respectively. Treating cyanocobalamin with chloramine-T, bromine water at pH 4, or N-chlorosuccinimide yields CNCbl–*c*-lactone, which can be reacted with an appropriate amine to generate an amide. Mild alkaline hydrolysis of the lactone gives the c-carboxylic acid. Mild acid hydrolysis of CNCbl yields a mixture of mono- and dicarboxylic acids derived from the *b*, *d*, and *e*-propionamide side chains. The acids can be separated and purified by chromatography on AG-1-X4 in the acetate form. The homogeneous acids are readily crystallized from aqueous acetone (17).

4.1 Diagnostic and Therapeutic Cobalamin Analogues

To be beneficial in the diagnosis and potential treatment of human disease, the modifications of cobalamin must not significantly alter its interaction with the transport proteins TC II and IF. Two currently available clinical assays—the unsaturated B_{12} binding capacity (UBBC) and intrinsic-factor binding assay (IFBA)—can assess the *in vitro* biological activity of newly synthesized cobalamin analogues. The binding of cobalamin analogues to IF is directly measured by the IFBA. Unfortunately, the UBBC assay does not separate TC II binding from HC binding; therefore, cobalamin analogue binding to TC II cannot be directly assessed by this assay.

Vu et al. have described the isolation and measurement of TC II from serum (18). However, the assay has yet to reach routine clinical utility. Recently, a recombinant human transcobalamin II (rh TC II) binding assay, as well as surface plasmon resonance, binding assays permit the direct quantification of new cobalamin bioconjugate TC II biological activity (6,19). Because the 5,6 dimethylbenzimidazole group is required for *in vivo* biological activity in humans, only modifications of the central cobalt ion, the carbon–cobalt bond of the beta ligand, and the *b*, *c*, *d*, and *e* side chains of the corrin ring will be discussed.

4.2 Cobalt Substitution

In August 1948, four months after B_{12} was isolated as a red crystalline compound from bovine liver extracts, emission spectrographic analysis showed that cobalt was a component of the vitamin (20). In early 1950, it was noted that microbial production of cobalamin could be increased by the addition of excess cobalt ion (21). Later that same year, Chaiet, Rosenblum, and Woodbury produced radioactive CNCbl by incubation of ^{60}Co with *Streptomyces griseus* cultures (22). Vitamin B_{12} was subsequently radiolabeled with ^{56}Co, ^{57}Co, and ^{58}Co over the next 10 years. However, due to either the long half-lives of the radioactive-cobalt-labeled Vitamin B_{12} analogues, or because of the energy of particle(s) emitted, the dose of these analogues was limited in human metabolic studies.

By 1960, ^{57}Co CNCbl became the preferred radiolabeled cobalamin analogue used for experimental and clinical studies (in the form of the Schilling test).

Numerous investigations of human and animal biodistribution ensued, including the evaluation of cobalamin uptake in neoplastic tissue. Due to the fear of radiation exposure, the maximum specific activity allowed for human studies was limited to 1 μCi/μg. (Theoretically, the maximum specific activity for ^{57}Co CNCbl is 300–330 μCi/μg.) (23) Thus, the low specific activity of the doses of ^{57}Co CNCbl that were administered eliminated the possibility of external imaging of TC II receptors by conventional gamma cameras.

4.3 Radioiodination of Cobalamin

As the interest in ^{57}Co CNCbl waned in the early 1970s, attempts were made to label cobalamin with a more suitable radionuclide. Initially, radioiodine was the most promising candidate. Unfortunately, imidazole and pyrazole are preferentially iodinated in the four position; therefore, fused ring compounds, such as the benzimidazole moiety of cobalamin, are not easily iodinated. To circumvent this problem, CNCbl was modified with tyrosine, histidine, or phenylalanine moieties on the acetamide or propionamide side chains for the purpose of iodination of the aromatic rings. These new bioconjugates labeled with ^{125}I and ^{131}I were intended to replace ^{57}Co CNCbl for *in vitro* measurements (24,25). However, the harsh conditions for labeling cobalamin with radioactive iodine, the low product yield, and suboptimal energy for *in vivo* imaging eventually limited the clinical applicability of the radioiodinated cobalamin bioconjugates.

4.4 Cobalamin–Chelator Bioconjugates

An analogy can be made to the clinically available somatostatin receptor-imaging agent Octreotide™. Originally, this somatostatin analogue with eight amino acids was labeled with ^{125}I at its single tyrosine residue for the *in vitro* verification of somatostatin receptors on neuroendocrine tumors. Later efforts toward *in vivo* imaging with ^{131}I- and ^{123}I-labeled somatostatin analogues were encouraging, but never came to fruition because of difficulties in radioiodinating the somatostatin analogue, as well as the instability of the C–I bond (with a dissociation energy of 60 kcal/mol). The latter problem resulted in significant free radioiodine due to the *in vivo* deiodination of the ^{131}I- and ^{123}I-labeled somatostatin analogue (26,27). Eventually, the octadentate chelator diethylenetriaminepentaacetate (DTPA) was covalently attached to the terminal phenylalanine residue of the analogue, allowing labeling with ^{111}In. Multiple clinical trials have shown the ^{111}In-DTPA somatostatin analogue to be highly effective for imaging neuroendocrine tumors (28).

Since rapidly proliferating tumors readily take up cobalamin, Collins and Hogenkamp (29) prepared several cobalamin-DTPA complexes. Reaction of the three monocarboxylic acids of cyano-, methyl- or adenosylcobalamin with 1,4-diaminobutane in the presence of the water-soluble carbodiimide, 1-ethyl-3(3′-dimethylaminopropyl) carbodiimide, and hydroxybenzotriazole gave the 4-aminobutyl amides of cobalamin. The aminobutyl-derivatized cobalamin was reacted with an excess of DTPA dianhydride to yield the desired cobalamin–DTPA complexes

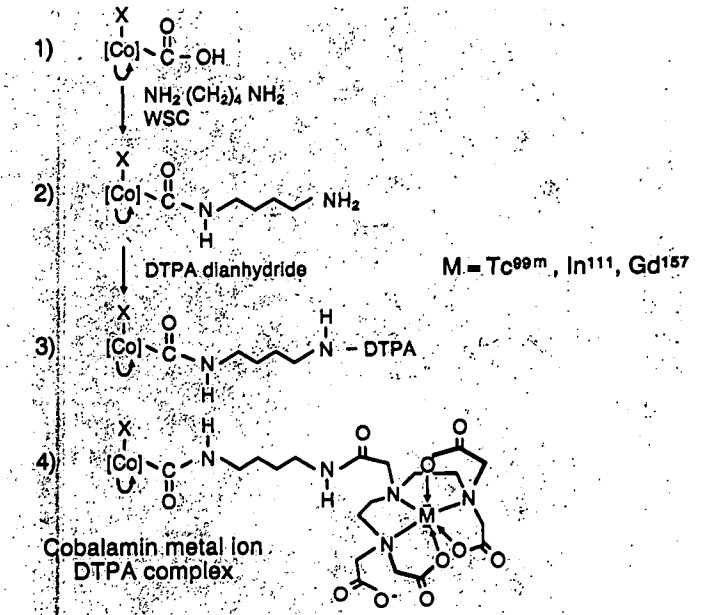
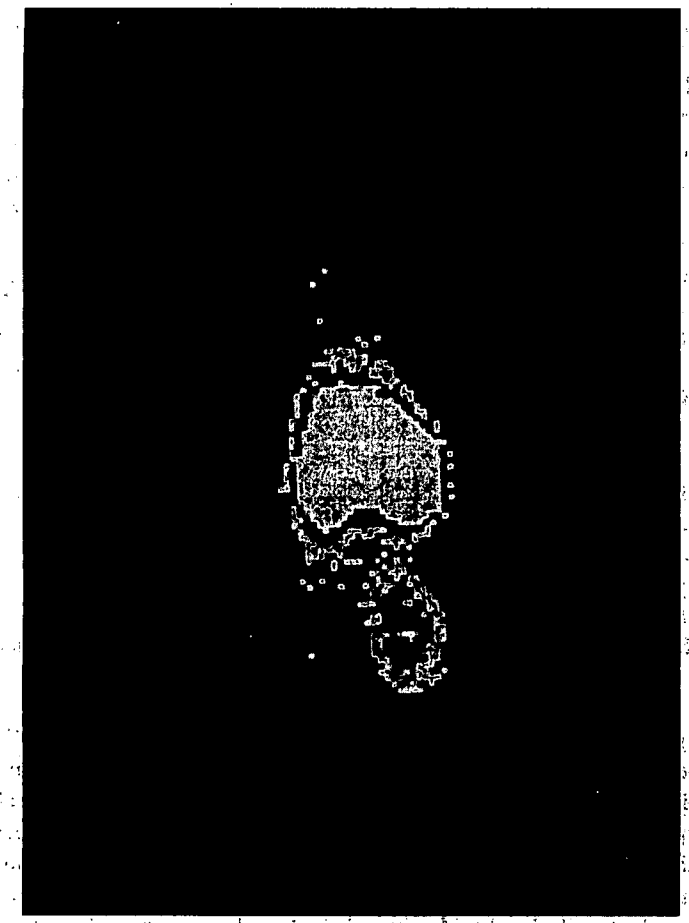


FIGURE 2 Synthesis of the cobalamin–diethylenetriaminepentaacetate complexes.

(Figure 2). These complexes were found to still interact with IF and TC *in vitro*, despite the rather major modification of the corrin ring. However, attachment of the chelator did lower the affinity to the carrier proteins—in particular, the affinity to IF. The specific activity of either ^{111}In- or ^{99m}Tc-labeled cobalamin analogue is approximately 300 μCi/μg (30), which has allowed TC II receptor imaging of normal and neoplastic tissue with AdoCbl–*b*-DTPA labeled with either ^{111}In or ^{99m}Tc. (See Figure 3.) Similarly, the MeCbl–*b*-DTPA–^{111}In analogue interacts with both the IF and TC II receptors *in vivo*, as demonstrated by the hepatic uptake at 24 hours after oral administration of the analogue (30). (See Figure 4.) Therefore, receptor-mediated diagnosis, as well as potential therapy, could be directed via TC II or IF receptors.

4.5 Gadolinium-Labeled Cobalamin

At the present time, ^{157}Gd–DTPA and other Gd(III) chelates are routinely used as paramagnetic- or magnetic-resonance-imaging (MRI) contrast enhancers. Gd(III) ion, containing seven unpaired electrons, is by far the most effective agent in accelerating proton relaxation. In the early 1980s, Weinmann et al. (31) showed that the chelation of Gd(III) with DTPA forms a very stable (k_a = 22), strong paramagnetic complex that is well tolerated in animals. These researchers also indicated that Gd–DTPA is restricted to the extracellular space and suggested that the hydrophilicity, charge, and molecular weight prevent intracellular uptake of the complex.



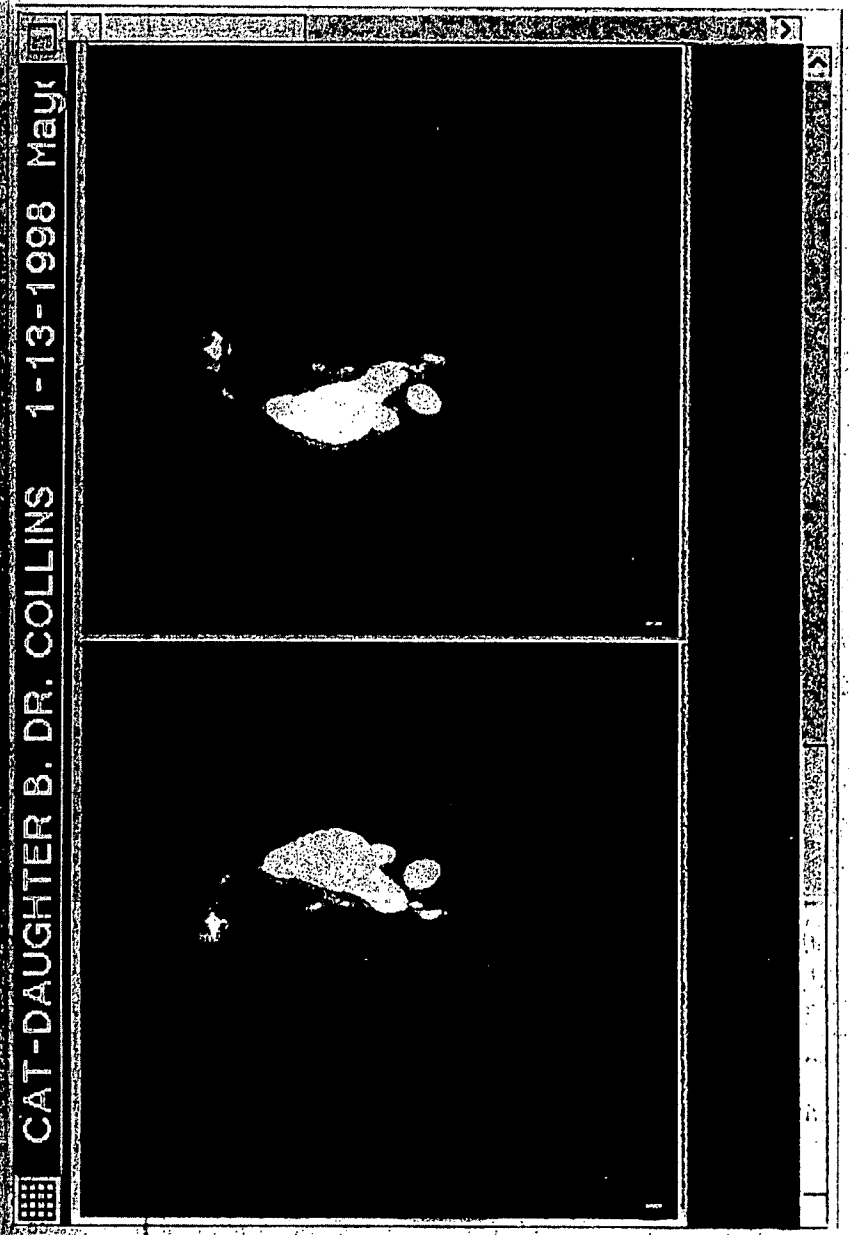


FIGURE 3 (A) A mouse burdened with human colon cancer (LS 174T) in the right flank was administered adenosyl–*b*-DTPA–^{111}In via injection into a vein in the tail. The image shown was obtained at 24 hours after injection. The mouse's nose is oriented at the top of the page. The large central white, red, and yellow areas represent the activity of the kidneys, GI tract, and liver, respectively. The smaller area of activity with a single speck of white surrounded by a larger region of red and yellow represents the colon tumor. (B) An eight-year-old female cat with a clinically palpable mammary carcinoma involving the entire left chain. The surpigenous activity along the abdominal wall represents biopsy-proven lobular carcinoma. The cat was injected with adenosylcobalamin–*b*-DTPA–^{99m}Tc via a vein in the forelimb. The images were obtained at 4 hours after injection. See color plate section.

FIGURE 3 (B) Continued

Receptor-mediated endocytosis (RME) is a specific, high-capacity process that can concentrate many molecules inside a cell in a short time. Receptor imaging via radiopharmaceutical compounds is more feasible than with paramagnetic agents, due to the lower concentration required within tissue to produce scintigraphic images. Specifically, nuclear medicine images can easily be obtained in concentrations as

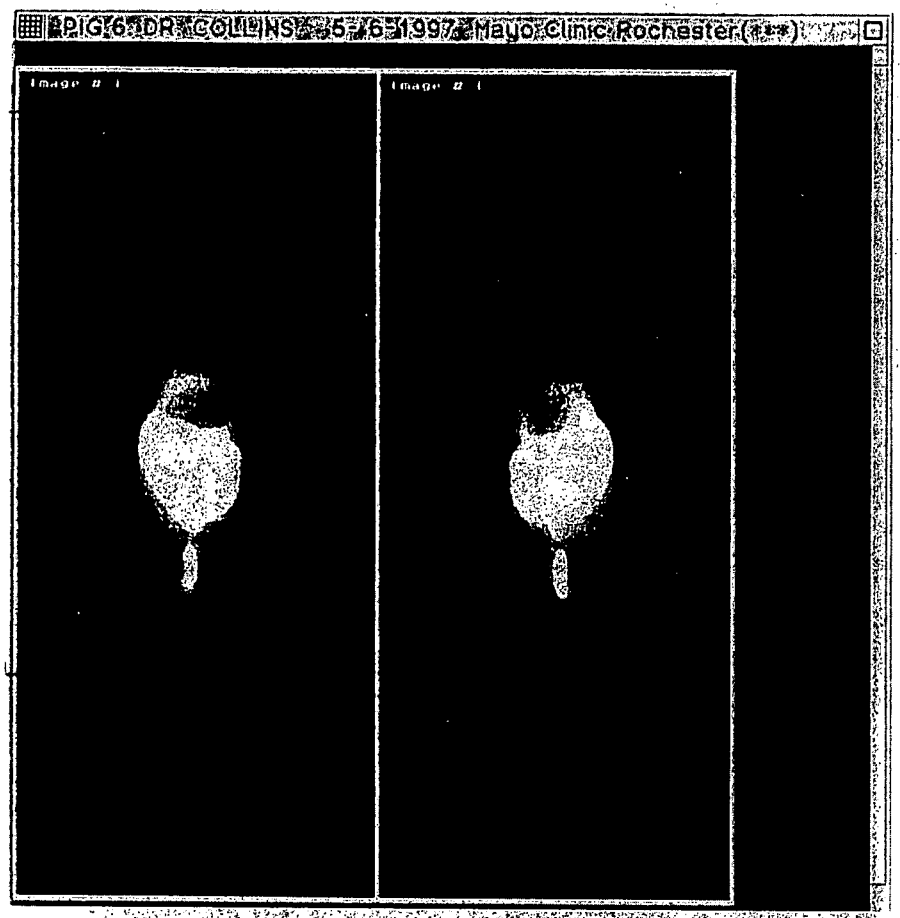


FIGURE 4 A 40-pound female pig with snout oriented at the top of the image. The pig was fed 2 μg of methylcobalamin–*b*-DTPA labeled with 600 μCi of ^{111}In. The images were obtained at 24 hours. The triangular-shaped area of activity in the right upper quadrant of the abdomen represents the liver. Central activity and linear activity near the tail represent radiolabeled cobalamin throughout the GI tract. See color plate section.

low as 1×10^{-14} M. By contrast, MRI images require a tissue concentration of 5.0×10^{-7} M of currently available Gd–DTPA contrast agent in order to induce a 50% increase in image enhancement using a standard T1 weighted spin echo pulse sequence in a 2-T magnet (32). Theoretically, the lowest concentration based on gadolinium dendrimers could move the minimal visible concentration down to 5.2×10^{-12} M. However, this concentration comes with the pharmacological implications of the appended large (140-kDa) dendrimer. Even with the minimum detectability of a theoretical sixth-generation relaxivity agent at 5.2×10^{-12} M, this is still two orders of magnitude weaker than the 2.8×10^{-14} M of Octreotide™ (M.W. ≈ 1.4 kDa) needed to image somatostatin receptors that are often upregulated in neuroendocrine tumors.

Although it might not be possible to image TC II and IF receptors via ^{157}Gd-labeled cobalamin–DTPA, the cobalamin analogues have several characteristics that may enhance the imaging of intravascular structures and various abdominal organs. The relaxivity of MeCbl and AdoCbl–DPTA–Gd complexes have been measured and compared with commercially available Magnevist™ and Prohance™. The latter paramagnetic agents are salts of DTPA chelating a single atom of ^{157}Gd. The *in vitro* relaxivity of the MeCbl and AdoCbl analogues was found to be two times greater than that of Magnevist™ or Prohance™ (33).

The ideal macromolecular MRI intravascular agent should have a distribution limited to the intravascular space, a sufficiently long blood half-life, and no immunogenicity. In addition, both the chelating agent and Gd(III) should be excreted completely. In order to lengthen the blood half-life and to increase the efficiency in proton relaxation enhancement, several macromolecules modified with DTPA have been synthesized. DTPA has been attached to proteins, polysaccharides, polylysine, and polyethyleneimine. For instance, Ogan and coworkers (34) prepared an albumin Gd-DTPA complex. An average of 19 Gd–DTPA chelates were attached to human serum albumin by reacting the protein with DTPA dianhydride. Unfortunately, this complex was later found to be immunogenic. Bogdanov et al. (35) prepared and evaluated a contrasting agent consisting of a monomethoxyether of poly(ethyleneglycol) attached to polylysine that was reacted with DTPA dianhydride. This complex was not immunogenic and its blood half-life was 14 hr.

The relaxivity of large macromolecules is greater than that of smaller gadolinium molecules due to the slower tumbling rate of the former in solution. Therefore, the cobalamin–DTPA–Gd complex should have greater relaxivity in solution compared with free Gd–DTPA. Conversely, if Gd–DTPA is bound to macromolecules or cell constituents or is in a high-viscosity environment, the normal tumbling of Gd–DTPA is slowed, which enhances its relaxivity. Therefore, binding to the 45-kDa TC II, as well as cell surface receptors, should improve the *in vivo* relaxivity of the cobalamin–DTPA–Gd agent.

In sum, the proprionamide side chains and the *c*-acetamide side chain are the most likely candidates for the development of radiopharmaceuticals or paramagnetic agents. Pathare et al. (14) have suggested that the Co-conjugated and ribose-conjugated derivatives are probably unsuitable for the purpose of synthesizing bioconjugates as diagnostic agents because of their photo- and biological instability (36,37). However, the light and enzymatic cleavage of these bioconjugates may render them useful in the treatment of malignancies. Additionally, the ability to manipulate the TC II receptor binding of cobalamin bioconjugates for either imaging or therapy via the preadministration of nitrous oxide, antifolates, or methionine-

deficient diet or blocking agents, or via the presaturation of TC II receptors with nonlabeled cobalamin, should further improve target-to-background ratios.

5 THERAPEUTIC USES OF COBALAMIN ANALOGUES

The ability to image a tumor with radiolabeled cobalamin suggests the potential to deliver therapeutic agents with similar selectivity. Because cobalamin is a precious micronutrient that occurs at extraordinarily low levels in most food, an elaborate system has evolved to harvest the vitamin, transport it to cells, recover unbound cobalamin from urine and feces, and reject degraded cobalamin fragments that could otherwise interfere with cellular metabolism. Cobalamin analogues and fragments can act as antimetabolites by blocking the normal utilization of cofactor. Alternatively, cobalamin can be used as a delivery vehicle by conjugating cobalamin and a therapeutic moiety. Both approaches will be discussed in the sections that follow.

5.1 Targeted Drug Delivery: General Considerations

Targeted drug delivery via "magic bullets" that seek cancer cells while sparing healthy cells has long been a goal of modern pharmacology (38). In practice, only partial selectivity has been achieved with traditional drugs, polymers, liposomes, and monoclonal antibodies (39). One of two problems is generally encountered: Either the drug reaches all of the target cells, but has an undesirable affinity for some nontargeted cells, or the drug reaches none of the nontargeted cells, but reaches only some of the targeted cells. In the first case, large amounts of drug may be required to produce a therapeutic effect, and collateral systemic toxicity may limit effective dosing. In the second case, incomplete delivery of the therapeutic agent to the target cells will leave some cells untreated, opening up the possibility of residual disease.

Temporal modulation of the drug concentration can also be used to improve targeting. Administration of a large dose of a nontherapeutic analogue can block the action of a drug that enters a cell only by receptor-mediated endocytosis. Theoretically, the uptake of a prodrug in a nontargeted tissue can be minimized if the target tissue has a greater number and turnover rate of receptors. As an example, compare the temporal distribution of a cytotoxic drug in cancer cells that express 1,000 receptors per cell, versus 50 receptors per normal cell. If 10,000 molecules per cell constitute a lethal dose from which the cell cannot recover, the cytotoxic threshold dose should be achieved faster in the cancer cell. If a large dose of a nontherapeutic analogue can be administered to saturate the smaller number and slower turnover rate of receptors on healthy cells, the excess cytotoxic drug could be flushed from the body via physiological clearance mechanisms. As a therapeutic agent or drug delivery vehicle, bioconjugates of cobalamin may benefit from the ability to flush excess drug via the administration of a bolus of authentic cobalamin, followed by renal and intestinal clearance of the excess cobalamin-based therapeutic drug.

Beyond analogues of cobalamin that might function as antimetabolites, cobalamin is useful as a general drug-delivery platform through the creation of cobalamin-drug bioconjugates. Both approaches will be discussed in the sections that follow. Other vitamin and micronutrient shuttle systems have been explored for their ability to deliver imaging and therapeutic agents to cells. Examples include folate, transferrin, ligands for the asialoglycoprotein receptor, riboflavin, thiamine, and biotin (40,41).

5.2 Depletion and Deprivation of Cobalamin

*5.2.1 **Depletion of Cobalamin with N_2O*** Dividing cells have an absolute requirement for cobalamin to support one-carbon metabolism prior to DNA synthesis. A logical strategy for treating cancer cells is to trigger cytostasis or apoptosis through the depletion and deprivation of cobalamin. L-1210 leukemia cells can achieve a peak cytoplasmic cobalamin concentration of about 25 nM with an extracellular cobalamin concentration of only 10 pM (37). Growth and replication of L-1210 require a minimum of 200 molecules of cobalamin per cell in culture, and replication ceases when the intracellular cobalamin concentration drops to less than 10 cobalamin molecules per cell (37,42).

The cobalamin-dependent growth of malignant tissue can be manipulated by the administration of N_2O. It is well known that body stores of cobalamin can be depleted after exposure to nitrous oxide for 24–72 hr. Cob(I)alamin that is transiently produced in the methionine synthase reaction can react with N_2O to form a catalytically inert cobalamin-containing holoenzyme (43,44). Combination therapy consisting of N_2O cobalamin depletion with the coadministration of traditional chemotherapeutic drugs offers some benefit in the treatment of leukemia and lymphoma, but the complete destruction of cobalamin stores precipitates the rapid onset of pernicious anemia and related metabolic disturbances (43).

*5.2.2 **Deprivation of Cobalamin with Antimetabolites*** Covalent dimers of cobalamin cannot serve as a bioavailable source of the cofactor, despite being recognized and transported by TC II (14,45). Antimetabolite dimers of cobalamin have been prepared for evaluation as potential antiproliferative agents against AIDS-related lymphoma (45). Two molecules of cobalamin can be cross-linked through the *b*-, *d*-, and *e*-propionate side chains with an intervening isophthalate spacer (45). The various cobalamin dimers are recognized by TC II with varying affinities and appear to be transported into the cell via receptor-mediated endocytosis. Once inside the cell, the cobalamin dimers block the synthesis of DNA precursors and inhibit cell replication. This therapeutic approach does not require the prior depletion of cobalamin stores, as rapidly dividing neoplastic cells will take up more of the inert cobalamin dimer than healthy cells will.

Cobalamin and cobalamin analogues can interfere with HIV-1 integrase, one of the enzymes required for the insertion of the human immunodeficiency virus into cellular DNA (46). The mechanism of inhibition is unknown, but Brouwer et al.

argue for a specific effect of cobalamin and against an earlier hypothesis which suggested that cobalamin might deplete NO (47). The IC_{50} values for hydroxocobalamin, methylcobalamin, and dicyanocobinamide are 1–7 μM against infection of cultured monocytes and 11–60 μM against infection of cultured lymphocytes. For even the best example of HIV-1 integrase inhibition by cobalamin, the concentration necessary to achieve 50% inhibition is at least two-orders of magnitude higher than the highest observed intracellular cobalamin concentration of 25 nM (37).

5.3 Bioconjugate Structure and Recognition by Transcobalamin

Recognition of the bioconjugate by TC II must be preserved to ensure transport. Cobalamin has many functional groups that are suitable for modification, including the β-axial ligand position at cobalt, the *b*-, *d*-, and *e*- side-chain amides (converted to the corresponding carboxylic acid for ease of derivatization), and the primary hydroxyl of the ribose. Covalent modification at any single position results in a unique cobalamin analogue with a corresponding binding affinity for TC II. Pathare et al. measured the general ability of TC II to bind a series of related cobalamin derivatives (14,45). (See Figure 1.)

It is not surprising to find the lowest specificity at the β-axial position of cobalt, since TC II must bind and transport a variety of cobalamins with ligands as diverse as hydroxide, cyanide, methyl, and 5′-deoxyadenosine. The next most accommodating position for derivatization is the primary hydroxyl of the nucleotide loop. Ligands that are covalently attached to cobalt can be released through mild photolysis, sonolysis, or intracellular enzymatic processes that interconvert cobalamins through the exchange of β-axial ligands. Therapeutic compounds that are covalently attached to the ribose hydroxyl must be released through degradative processes.

*5.3.1 **Binding Quantification by Surface Plasmon Resonance*** The cellular uptake of a radiolabeled cobalamin antimetabolite or drug-cobalamin analogue can be estimated by the use of radiolabeled cobalamin. Recognition by TC I or TC II can be quantitatively described *in situ* by surface plasmon resonance in a nonradioactive competitive binding assay. A solution of cobalamin-binding protein (either TC I, or TC II, or both) and the cobalamin analogue to be evaluated flows through a Biacore™ assay chip that has been derivatized with a cobalamin analogue. The cobalamin analyte competes with immobilized cobalamin for TC I and TC II, to produce an angle of deflection of the evanescent wave that is proportional to the change in the amount of surface-bound protein.

As a demonstration of this approach, Myszka, Grissom, and West immobilized hydroxocob(II)alamin-*b*-(4-aminobutylamine) onto a CM5 carboxymethyldextran surface at a level of 1,300 response units, using standard amine coupling chemistry with carbodiimide (48,49). Binding experiments were carried out by passing a preequilibrated mixture of porcine TC I and TC II (called "nonintrinsic factor" by Sigma, Inc.) and ligand over the derivatized surface. The initial rate of binding to the surface was monitored as a change in RU per second. After each experiment the surface was regenerated with a 10-s pulse of guanidine.

The binding of adenosylcobalamin, cyanocobalamin, hydroxocobalamin, methylcobalamin, and six-diethylene–triaminepentaacetate (DTPA)–cobalamin imaging agents was evaluated at a total protein concentration of 0.2 μg/mL and various ligand concentrations from 0.01–100 nM. The response from a mixture of TC I and TC II in the absence of ligand was 0.655 response units, with highly reproducible values. Figure 5 compares the binding inhibition caused by 1-nM ligand with values related to the maximum possible loading of protein in the absence of free ligand. Lower values indicate greater recognition by the mixture of TC I and TC II. The data in the figure show that MeCbl–*d*-(4-aminobutyl) amide–DTPA, MeCbl–*e*-(4-aminobutyl) amide–DTPA, and AdoCbl–*d*-(4-aminobutyl) amide–DTPA are not recognized by the mixture of TC I and TC II as well as it recognizes the authentic cofactors, or AdoCbl–*b*-(4-aminobutyl) amide–DTPA, or MeCbl-*d*–(4-aminobutyl) amide–DTPA. This experiment shows surface plasmon resonance to be a useful technique for assessing the recognition of cobalamin bioconjugates by TC I and TC II.

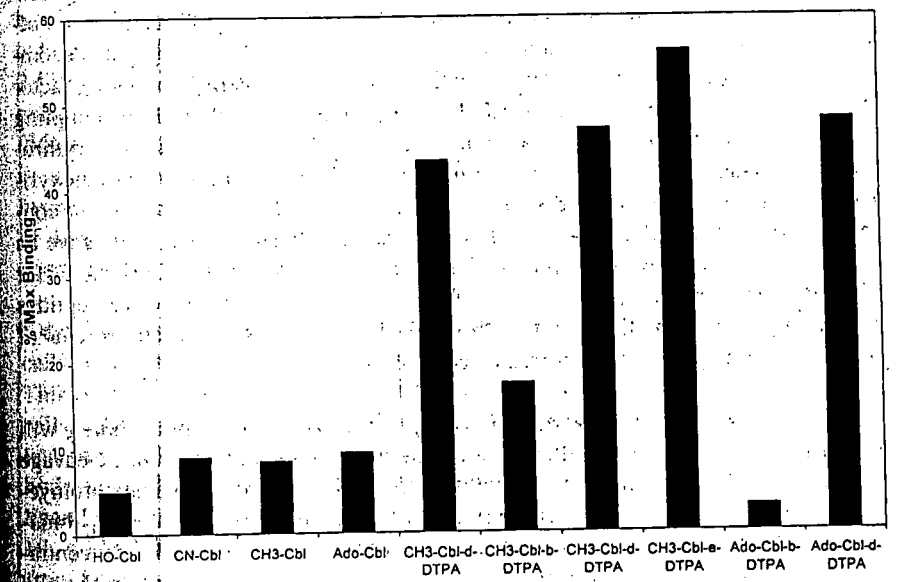


FIGURE 5 The response from a mixture of TC-I and TC-II in the absence of ligand was 0.655 response units. Binding inhibition is caused by 1-nM ligand, with values compared to the maximum possible loading of protein in the absence of free ligand. Lower values indicate greater recognition by the mixture of TC-I and TC-II. Abbreviations are as follows: methylcob(III)alamin, CH_3–Cbl; 5′deoxyadenosylcob(III)alamin, Ado–Cbl; cyanocob(III)alamin, CN–Cbl; methylcob(III)alamin–*d*-(4-aminobutyl) amide–DTPA, CH_3–Cbl–*d*-DTPA; methylcob(III)alamin–*e*-(4-aminobutyl) amide–DTPA, CH_3–Cbl–*e*-DTPA; adenosylcob(III)alamin–*d*-(4-aminobutyl) amide–DTPA, Ado–Cbl–*d*-DTPA; and adenosylcob(III)alamin–*b*-(4-aminobutyl) amide–DTPA, Ado–Cbl–*b*-DTPA.

5.4 Delivery of Therapeutic Agents

Cobalamin has been used to enhance the oral bioavailability of tethered peptides and proteins, as well as to target the delivery of intravenously administered cytotoxic chemotherapeutic agents. Biochemical instability and inefficient transport across the villous epithelium often limits the successful oral delivery of peptides and many small molecules. Conjugation to cobalamin offers the hope of increasing the bioavailability of peptides and drugs that have poor solubility and low intestinal absorption. Activation of the prodrug must occur following translocation to the serum or uptake by the target cell. Thus, a combination of biological stability and temporal or spatial lability must be designed into cobalamin conjugates in order to have a therapeutically useful vehicle for oral bioavailability or cell-targeted delivery.

5.4.1 Oral Delivery Granulocyte-colony stimulating factor, erythropoietin, and luteinizing hormone-releasing hormone have been conjugated to cobalamin in an attempt to achieve orally active formulations of these peptide hormones (50,51). Increased oral bioavailability was achieved by conjugation of a buried thiol in granulocyte-colony stimulating factor with a dithiopyridyl derivative of cobalamin. Following translocation from the gut to the circulatory system, the bioconjugate decomposes via serum glutathione to yield bioactive granulocyte-colony stimulating factor (50). Conjugates of erythropoietin (EPO) were prepared by carbodiimide coupling chemistry to link the C-terminus carboxyl group or Asp/Glu residues of erythropoietin to a 2-aminoethyl-*e*-cobalamin analogue and to (6-amino-3,4-dithiahexyl)-*e*-cobalamin. This approach led to unwanted cobalamin dimer formation, but conjugates formed via hydrazido- and adiphylhydrazidocobalamin precursors gave an acceptable yield of active bioconjugate and were well recognized by intrinsic factor (50). This approach is a promising one for the oral delivery of bioactive peptides that are required in small amounts. EPO at 51 kDa suggests that even proteins of moderate size may be cotransported as cobalamin bioconjugates.

5.4.2 Targeted Delivery of Cytotoxic Drugs Ideally, a cobalamin prodrug will be transported into a cancer cell, where it remains biologically inert until cleavage of the covalent linker releases the active drug (52). (See Figure 6.) In acute promyelocytic leukemia, a three- to twenty-six fold increase in the unsaturated B_{12} binding capacity of blood is observed, which is related to an increase in the concentration of the B_{12} binding proteins TC I and TC-II (53,54). Some patients with solid tumors also exhibit an increase in TC I and TC II levels (55). The increase in unsaturated serum cobalamin-binding capacity is presumed to correspond to an increased demand for cobalamin by rapidly dividing cells.

5.4.3 Synthesis of Drug–Cobalamin Bioconjugates The nitrogen mustard chlorambucil was selected for conjugation to cobalamin (56). This bifunctional alkylating agent is known to cross-link DNA by reaction of guanosine at N-7 and thereby impair cell replication. A two-carbon hydroxyl linker was inserted between the

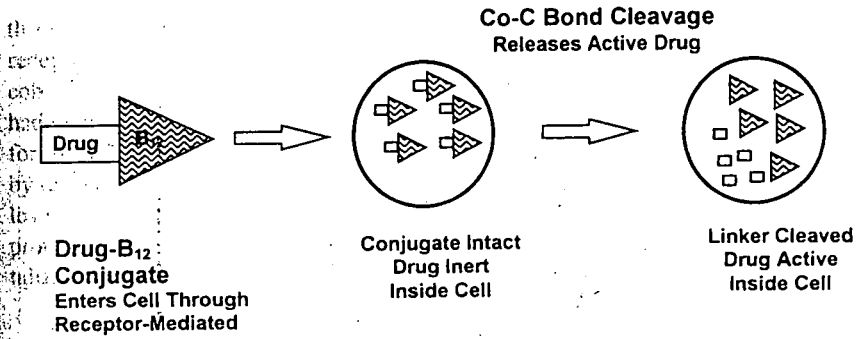


FIGURE 6 The inactive drug–cobalamin bioconjugate enters cancer cells through receptor-mediated endocytosis. Once the biconjugate is inside the cell, the linker between cobalamin and the drug is cleaved through a process that may be enzymatic or may depend upon the redox state or pH of the intracellular environment.

cobalt atom of cobalamin and the carboxylate of chlorambucil (56). The chlorambucil–hydroxyethyl–cobalamin bioconjugate was produced by combining aqueous cob(I)alamin (produced by $NaBH_4$ reduction of aquocob(III)alamin) with 1-bromo-2-[4-[4′-[bis(2-chloroethyl) amino]phenylbutyroxy] ethane to give 2-[4-[4′-[bis(2-chloroethyl)amino]-phenyl]butyroxy]ethylcob(III)alamin (hereafter, Chl–HE–Cbl) (56). (See Figure 7.) The hydroxyethyl linker between chlorambucil and cobalamin is metastable in aqueous solution and decomposes to yield free chlorambucil and hydroxocobalamin with $t_{1/2}$ = 70 min at 25° C. Biological results suggest that the rate of decomposition may be slowed in blood. The process responsible for decreased decomposition is not yet known.

5.4.4 Assay of Cytotoxicity Against HL-60 Leukemia Cells Murine leukemia cells (L-1210) attain intracellular cobalamin concentrations of at least 25 nM (34). This level is significantly higher than the concentration reached in most somatic cells, other than the liver and the proximal tubules of the kidney (2). Furthermore, leukemia cells are known to actively interconvert the β-axial ligand from cyano-, hydroxo-, adenosyl-, and methylcobalamin (34). Interconversion of the β-axial ligand of a drug–cobalamin complex will release the attached cytotoxic drug. The HL-60 leukemia cell line was chosen as a test system, since this strain is derived from humans with promyelocytic leukemia and it is the form of leukemia with the greatest increase in unsaturated serum cobalamin-binding capacity (53). The ability of Chl–HE–Cbl to kill HL-60 human leukemia cells grown in culture was quantified by a cell viability assay that is based on the metabolism of 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium thiazolyl bromide (MTT) to a purple formazan dye through the action of alcohol dehydrogenase (57,58). Figure 8 shows the results of a test of Chl–HE–Cbl and free chlorambucil against HL-60. In this cell line, the unconjugated chlorambucil standard exhibits an LC_{50} of 3 μM. A potential therapeutic benefit has been realized, as greatly reduced toxicity is observed if other cells

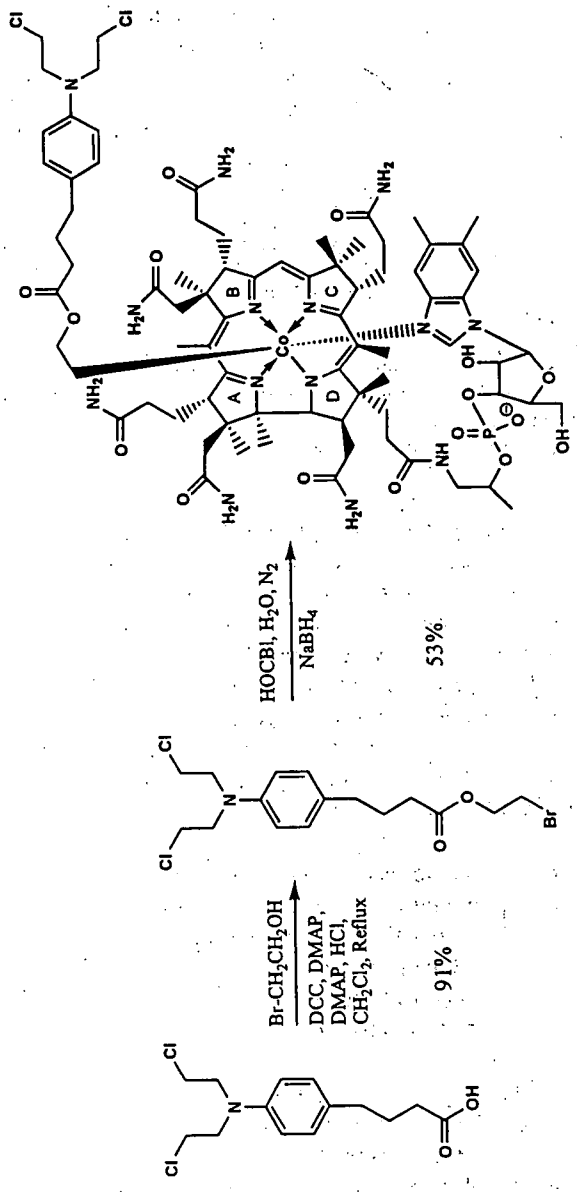


FIGURE 7 Synthesis of chlorambucil–hydroxyethyl–cobalamin (Chl–HE–Cbl) (55).

throughout the body do not activate the prodrug. Figure 8 also shows that the receptor-mediated uptake of Chl–HE–Cbl can be blocked by saturation of the cobalamin receptor with 10 equivalents of HO–Cbl. If the release of the active drug had occurred primarily outside of the cell, the LC_{50} would have been the same as for free drug. The HL-60 leukemia cell line has the ability to release the active drug by exchanging the β-ligand at cobalt. Targeted drug delivery with cobalamin, followed by intracellular activation in leukemia cells, merits further investigation as a promising new method of selectively delivering chemotherapeutic drugs to immortalized cells of the hematopoietic system. Further investigation of the intracellular

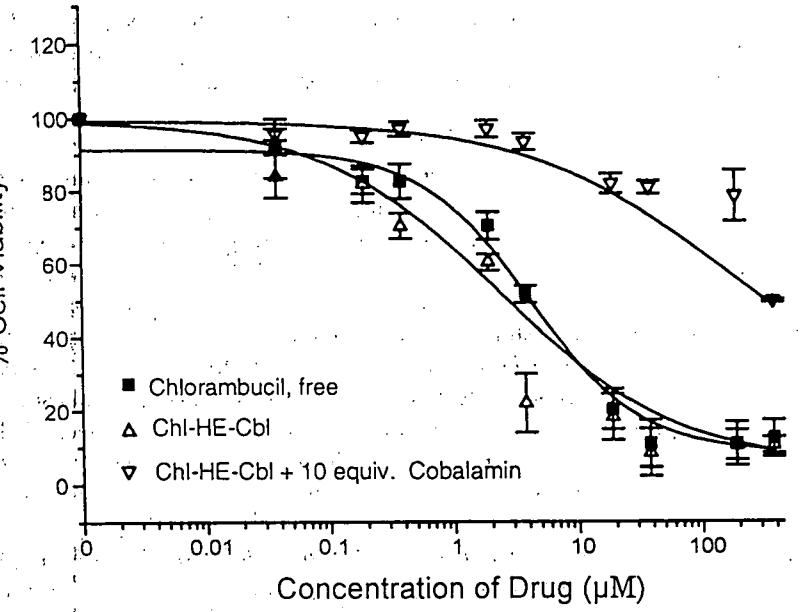


FIGURE 8 *In vitro* effectiveness of chlorambucil–hydroxyethyl–cobalamin bioconjugate against the HL-60 human promyelocytic leukemia cell line (58). The percentage of cell viability is determined by an MTT assay of live cells three days after treatment with the drug or bioconjugate. The concentration of free chlorambucil, Chl–HE–Cbl, or Chl–HE–Cbl plus 10 equivalents of HO–Cbl is varied over a range of 10^6 HL-60 cells from ATCC were grown in α-MEM media at 37° C and 5% CO_2. The cells were dispersed in a round-bottom 96-well microtiter plate at a density of 25,000 cells per well and a volume of 200 μL. The cells were centrifuged after 24 hr, and the supernatant was carefully aspirated without disturbing the pellet. A 200-μL aliquot of fresh α-MEM medium was added to each well, and the cells were returned to the incubator for 48 hr. Again, the culture medium was removed by aspiration following centrifugation, and 100 μL of medium containing MTT dye was added to each well. After 3 hr of incubation at 37° C, the cells were lysed by the addition of 1.2 M HCl in 60% ethanol, and the absorbance at 405 nm was measured for each well with the use of a BIO-RAD microplate reader. Values of 100% cell viability were determined by the control experiments in which no drug or Chl–HE–Cbl was added to the well (58).

β-ligand exchange process is required to differentiate an enzymatic process from a nucleophile- or glutathione-dependent process. Decomposition of the hydroxyethyl linker occurs with a half-life of 70 min at 25° C and must also contribute to the release of active chlorambucil, but the rate of this cleavage process when the cobalamin conjugate is bound to TC I and TC II is unknown.

5.4.5 Externally Triggered Drug Activation (Release) In cells that do not spontaneously cleave the Co–C bond, or for prodrugs that do not undergo time-dependent activation, an external trigger must be employed to release the cytotoxic drug. Because the Co–C bond has an unusually low dissociation energy of 31–37 kcal/mol, visible light can be used to photocleave the cobalamin–drug bioconjugate within cells. Reductive and oxidative products derived from the sonolysis of water can also be used to cleave the bond. Both methods allow the selective activation of a cytotoxic drug–cobalamin bioconjugate by the application of an extracorporeal signal (56).

5.4.6 Light-Triggered Drug Activation The $\pi \rightarrow \pi^*$ electronic transition of the corrin ring of cobalamin produces a long-wavelength absorption maximum at 525 nm. Irradiation of alkylcobalamins at this wavelength leads to cleavage of the Co–C bond with a photolysis quantum yield of 0.1–0.3. The ability to use green light in the range of 500-590 nm to activate a cobalamin prodrug under tissue culture conditions has been demonstrated. However, human tissue becomes translucent to red light only in the range of 600–750 nm, wherein minimal light is absorbed and the intensity is diminished solely by scattering (59,60). Therapeutic applications that demand deep penetration of light would require the use of a red-shifted cobalamin analogue to enhance tissue penetration. At least one analogue with extended conjugation in the corrin ring has been described (61).

5.4.7 Assay of Cytotoxicity against HCT-116 Colon Tumor Cells with Light Activation The effectiveness of the (Chl–HE–Cbl) bioconjugate has been evaluated against the HCT-116 colon tumor cell line *in vitro*, with the results shown in Figure 9. Authentic chlorambucil (black squares) has an LC_{50} of about 20 μM, whereas Chl–HE–Cbl shows no substantial toxicity at concentrations approaching 1 mM (triangles). If cells treated with Cbl–HE–Cbl are subjected to brief photolysis three hours after dosing, the LC_{50} decreases by an order of magnitude, to 1 μM (circles). If 10 equivalents of HO–Cbl are added to saturate the TC–cobalamin receptors, Chl–HE–Cbl is not taken into the cells, and photolysis triggers the release of active chlorambucil in the cell culture medium. The released chlorambucil now enters the cell by passive diffusion, and an LC_{50} of 20 μM is observed, in close agreement with the value for the chlorambucil standard (diamonds).

5.4.8 Ultrasound-Triggered Drug Release Cobalamin bioconjugates are unique among all prodrugs in that they can also be activated by ultrasound to cleave the C–Co bond, thereby allowing the drug to be released deep within tissue that is not as easily accessible with light. A detailed description of the sonolysis of chlorambucil–cobalamin bioconjugates has been published (56).

5.4.9 The Chemistry of Ultrasound Ultrasound irradiation and sonochemistry are often not described as high-energy processes, but during sonolysis, the devel-

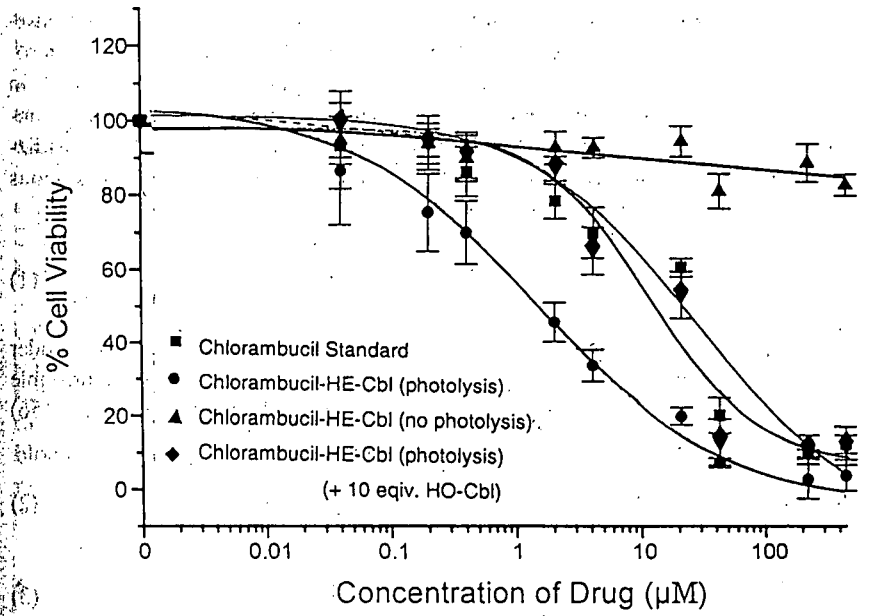


FIGURE 9 *In vitro* effectiveness of chlorambucil–hydroxyethyl–cobalamin bioconjugate against the HCT-116 human colon tumor cell line (58). The percentage of viable cells, as indicated by the colorimetric dye 3-[4,5-diamethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT), is measured three days after treatment with Chl–HE–Cbl and with or without photolysis or added hydroxocobalamin, as indicated. The percentage of cell viability is plotted as a function of the drug or bioconjugate concentration in the assay well. The drug bioconjugate is diluted over five orders of magnitude (approximately 0.005 to 50 μg/mL). Four hours after seeding of the cells in the plate, the cells are treated with the appropriate drug, dissolved in isotonic buffered solution. In control experiments without photolysis-triggered drug release, the compound is left on the cells for three days. In the wells for triggered drug release, light from a high-intensity green light-emitting diode is focused on selected wells for five minutes. The cells are incubated in a 5% CO_2-balanced atmosphere at 37°C. After three days, the cells are refed and MTT is added. The reduction of MTT to purple formazan product is quantified in a 96-well plate reader. The concentration of the purple formazan dye correlates with the number of viable cells. The reduction in cell survival at a given dose and photolysis exposure gives a quantitative estimation of cell death and the effectiveness of drug delivery. Care was taken not to expose portions of the plate to photolysis conditions through the adventitious spillover of radiation. This is accomplished by using 96 well plates with an opaque mask.

opment, growth, and implosion of bubbles in a liquid create extreme reaction environments on a microscopic scale. The collapse of cavitation bubbles produces microscopic pressures in excess of 500 atmospheres and microscopic temperatures in excess of 5,000° C (61). The sonolysis of aqueous solutions produces a high concentration of hydroxyl radicals and hydrogen atoms (63–65). These reactive oxidizing and reducing species are responsible for initiating most reactions in aqueous solvents, as shown (65) by the equation

$$\text{H–OH} \xrightarrow{)))))} \text{H}\bullet + \bullet\text{OH} \qquad (1)$$

As an example, consider the effect of aqueous sonolysis on methylcobalamin. Under anaerobic conditions, the hydrogen atom can reduce methylcobalamin to an unstable 19-electron species that decomposes to liberate cob(II)alamin and methane (56) according to the following reactions:

$$\text{H}\bullet + \text{CH}_3\text{–Cbl}^{\text{III}} \rightarrow \text{CH}_3\text{–Cbl}^{\text{II}-} + \text{H}^+ \qquad (2)$$

$$\text{CH}_3\text{–Cbl}^{\text{II}-} \rightarrow \text{Cbl}^{\text{II}} + \text{CH}_3^- \qquad (3)$$

$$\text{CH}_3^- + \text{H}^+ \rightarrow \text{CH}_4 \qquad (4)$$

The formation of hydroxyl radicals *in vivo* has been the focus of several investigations because of the potentially deleterious effects of oxidizing free radicals in human tissue (66,67). Recently, focused ultrasound has been used *in vivo* to activate Photofrin™ and related photodynamic drugs deep within tissue (68). The promise of focused ultrasound for promoting the transvascular transport and delivery of tumor-imaging agents and drugs has also been recognized (69). Ultrasonic equipment ranging in frequency from 15 kHz to 10 MHz will produce radicals even in microsecond pulses (70,71). The radicals that are formed do not appear to present an unacceptable health risk, as clinical experience tells us that diagnostic ultrasound is a benign procedure. However, it is the ability to form these radicals by sonolysis *in vivo* that allows the triggered release of drug molecules from drug–cobalamin and drug–corrinoid bioconjugates (56).

5.4.10 Removal of Unactivated Drug–Cobalamin Bioconjugate A significant advantage of cobalamin–drug bioconjugates is the ability to remove residual unactivated prodrug from a patient following treatment. Because cobalamin is a water-soluble vitamin, transport and storage constitute a dynamic process wherein cobalamin is recovered from the urine by the kidneys and returned to the bloodstream through saturable receptors in the glomerulus (37). Furthermore, a significant fraction of the daily requirement of cobalamin is excreted via bile into the gut only to be reabsorbed by the intestine as the IF–cobalamin complex. This constant

turnover of cobalamin allows for the removal of unactivated drug–cobalamin prodrug by administering a large intravenous or oral "flushing" dose of natural vitamin B_{12} after treatment with light or ultrasound. This dose will saturate cobalamin receptors in the liver and kidney and cause the remaining unactivated prodrug to be excreted into the urine and feces. The ability to saturate cobalamin receptors *in vivo* and "flush" excess vitamin B_{12} into the urine and feces, first performed by Schilling (72), was recently demonstrated with the cobalamin–DTPa bioconjugates (29,30). Such saturation should allow patients who are given a photolabile cobalamin–drug bioconjugate to return to normal light exposure within 12–24 hours after treatment.

Although the liver is a major site of cobalamin storage, hepatic uptake of drug–cobalamin bioconjugates appears not to be a problem. Transcobalamin I is a glycoprotein that binds cobalamin in the blood and protects it against photolysis by visible light that penetrates tissue. The asialoglycoprotein receptor on liver cells appears to bind glycosylated cobalamin–TC 1 complex and internalizes cobalamin from the blood for hepatic storage and processing, but the process occurs at a much slower rate than the uptake of cobalamin by tumor cells (2).

6 CONCLUDING REMARKS

Despite its structural complexity, cobalamin can be selectively modified at a variety of sites. This selective modification has allowed for the systematic attachment of a variety of molecules for cellular delivery, as well as permitting enhanced oral delivery of peptides and proteins via IF-mediated uptake. Since proliferating tissues take up proportionately greater amounts of cobalamin, the possibility exists for tissue-selective delivery of both imaging agents and cytotoxic drugs via new cobalamin bioconjugates. Because this subfield of cobalamin chemistry is still in its infancy, we may expect many exciting advances in the near future.

REFERENCES

1. Hall, C. A. (1984), *J. Lab. Clin. Med.* **103**, 70–81.
2. Flodh, H. (1968), *Acta Radiological Supplement* **284**, 3–80.
3. Allen, R. H. (1975), *Prog. Hematol.* **9**, 57–84.
4. Donaldson, R. M. (1987), in *Physiologic of the Gastrointestinal Tract*, 2d ed. (Johnson, L. R., Alpers, D. H., Christensen J., Jacobsen, E. D., and Walsh, J. H., eds.), pp. 959–973, Raven Press, New York.
5. Seetharram, B. (1994), in *Physiology of the Gastrointestinal Tract*, Vol. 2, 3d ed. (Johnson, L. R., Alperts, D. H., Christensen, J., Jacobsen, E. D., and Walsh, J. H., eds.), pp. 1997–2026, Raven Press, New York.
6. Dan, N., and Cutler, D. F. (1994), *J. Biol. Chem.* **269**, 18849–18855.
7. Hall, C. A. (1990), in *Biomedicine and Physiology of Vitamin B_{12}* (Linnell, J. C. and Bhatt, H. R., eds.), pp. 239–253, The Children's Medical Charity, London.

8. Moestrup, S. K. (1998), in *Vitamin B_{12} and B_{12} Proteins* (Krautler, B., Arigoni, D, and Golding, B. T., eds.), pp. 477–489, Wiley-VCH, Weinheim, Germany.
9. Moestrup, S. K., Birn, H., Fischer, P. B., Petersen, C. M., Verroust, P. J., Sim, R. B., Christensen, E. I., and Nexo, E. (1996), *Proc. Natl. Acad. Sci.* **93**, 8612–8617.
10. Moestrup, S. K. (1994), *Biochim. Biophys. Acta.* **1197**, 197–213.
11. Jacobsen, D. W., Amagasaki, T., and Green, R. (1990), in *Biomedicine and Physiology of Vitamin B_{12}* (Linnell, J. C. and Bhatt, H. R., eds.) pp. 293–306, The Children's Medical Charity, London.
12. McLean, G. R., Quadros, E. V., Rothenberg, S. P., Morgan A. C., Schroder, J. W., and Ziltner, H. J., (1997), *Blood* **89**, 235–242.
13. Toraya, T., Ohsahi, K., Meno, H., and Fukui, S. (1975), *Bioinorg. Chem.* **4**, 245–255.
14. Pathare, P. M., Wilbur, D. S., Heusser, S., Quadros, E. V., McLoughlin, P., and Morgan, A. C. (1996), *Bioconjugate Chem.* **7**, 217–232.
15. Takaheta, Y., Mishizawa, A., Kojima, I., Yamanishi, M., and Toraya, T. (1995), *J. Nutr. Sci. Vitaminol.* **41**, 515–526.
16. Olesen, J., Hippe, E., and Haber, E. (1971), *Biochim. Biophys. Acta.* **243**, 66–74.
17. Anton, D. L., Hogenkamp, H. P. C., Walker, T. E., and Matwiyoff, M. A. (1980), *J. Amer. Chem. Soc.* **102**, 2215–2219.
18. Vu, T., Amin, J., Ramos, M., Flener, V., Vanyo, L., and Tisman, G. (1993), *Am. J. Hematol.* **42**, 202–211.
19. Quadros, E. V., Sai, P., and Rothenberg, S. P. (1993), *Blood* **81**, 1239–1245.
20. Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K. (1948), *Science* **108**, 134.
21. Hendlin, D. and Ruger M. L. (1950), *Science* **111**, 451–542.
22. Chaiet, L., Rosenblum, C., and Woodbury, D. T. (1950), *Science* **111**, 601–602.
23. Rosenblum, C. (1966), in *Radioactive Pharmaceuticals* (Andrews, G. A., ed.), Chapter 26, USAEC, Washington, DC.
24. Endres, D. B., Dainter, K., and Niswender, G. D. (1978), *Clin. Chem.* **24**, 460–465.
25. Houts, T. M. (1982), *Clinca Chimica Acta* **126**, 315–322.
26. O'Connor, M. K., Kvols, L. K., Brown, M. L., Hung, J. C., Hayostek, T. J., Cho, D. S., and Vetter, R. J. (1992), *J. Nucl. Med.* **33**, 1613–1619.
27. Krenning, E. P., Bakker, W. H., Kooij, P. P. M., Breeman, W. A. P., Oei, H. Y., de Jong, M., Reubi, J. C., Visser, T. J., Bruns, C., Kwekkeboom, D. J., Rejis, A. E. M., van Hagen, P. M. Koper, J. W., and Lamberts, S. W. J. (1992), *J. Nucl. Med.* **33**, 652–658.
28. Krenning, E. P., Kwekkeboom, D. J., Bakker, W. H., Breeman, W. A. P., Kooij, P. P. M., Oei, H. Y., van Hagen, M., Postema, P. T. E., de Jong, M., Reubi, J. C., Visser, T. J., Reijs, A. E. M., Hofland, L. J., Koper, J. W., and Lamberts, S. W. J. (1993), *Eur. J. Nucl. Med.* **20**, 716–731.
29. Collins, D. A. and Hogenkamp, H. P. C. (1997), *J. Nucl. Med.* **38**, 717–723.
30. Collins, D. A., personal communication, manuscript in preparation.
31. Weinmann, H.-J., Brasch, R. C., Press, W.-R, and Wesbey, G. E. (1984), *Amer. J. Roentegen.* **142**, 619–624.
32. Nunn, A. D., Linder, K. E., Tweedle, M. F. (1997), *Nucl. Med.* **41**, 155–162.
33. Hogenkamp, H. P. C. and Collins, D. A. (1996), *Biofactors* **5**, 180–182.

34. Ogan, M. D., Schmiedl, M., Moseley, H. E., Grodd, W., Parajanen, H., and Brasch, R. C. (1987), *Invest. Radiol.* **22**, 665–671.
35. Bogdanov, A. A., Weissleder, R., Frank, H. W., Bogdanova, A. V., Nossif, N., Schaffer, B. K., Tsai, E., Papisov, M. I., and Brady, T. J. (1993), *Radiol.* **187**, 701–706.
36. Quadros, E. V., Jackson, B., Hoffbrand, A. V., and Linnell, J. C., *Vitamin B_{12}* (B. Zagalak and W. Friedrich, eds.), pp. 1045–1054, Walter de Gruyter & Co., New York (1979).
37. Quadros, E. V. and Jacobsen, D. W. (1995), *Biochim. Biophys. Acta* **1244**, 395–403.
38. Ehrlich, P. (1909), *Chem. Ber.* **42**, 17–20.
39. Langer, R. (1998), *Nature* **392**, 5–10.
40. Low, P. S. (1995), *Restorative Neurology and Neuroscience* **8**, 15–16.
41. Leamon, C. P. and Low, P. S. (1991), *Proc. Natl. Acad. Sci. USA* **88**, 5572–5576.
42. Fujii, K. and Woods, D. D. (1981), *Biochem. J.* **256**, 10329.
43. Abels, J., Kroes, A. C. M., Ermens, A. A. M., van Kapel, J., Schoester, M., Spijkers, L. J. M., and Lindemans, J. (1990), *Amer. J. Hematol.* **34**, 128–131.
44. Drummond, J. T. and Matthews, R. G. (1994), *Biochemistry* **33**, 3732–3741.
45. Pathare, P. M., Wilbur, D. S., Hamlin, D. K., Heusser, S., Quadros, E. V., McLoughlin, P., and Morgan, A. C. (1997), *Bioconj. Chem.* **8**, 161–172.
46. Weinberg, J. B., Shugars, D. C., Sherman, P. A., Sauls, D. L., and Fyfe, J. A. (1998), *Biochem. Biophys. Res. Commun.* **246**, 393–397.
47. Brouwer, M., Chamulitrat, W., Ferruzzi, G., Sauls, D. L., and Weinberg, J. B. (1996), *Blood* **88**, 1857–1864.
48. Myszke, D., Grissom, C. B., West, F. G., unpublished results.
49. Chaiken, I., Rose, S., and Karlsson, R. (1992), *Anal. Biochem.* **201**, 197.
50. Russell-Jones, G. J., Westwood, S. W., and Habberfield, A. D. (1995), *Bioconj. Chem.* **6**, 459–465.
51. Russell-Jones, G. J., Westwood, S. W., Farnworth, J. K., Findlay, J. K., and Burger, H. G. (1995), *Bioconj. Chem.* **6**, 34–42.
52. A preliminary report of this work was presented at the American Chemical Society meeting, Las Vegas, Nevada, Sept. 8, 1997.
53. Schneider, Z., and Stroinski, A. (1987), *Comprehensive B_{12}*, p. 358, de Gruyter, Berlin.
54. Rachmilewitz, B., Rachmilewitz, M., Moshkowitz, B., and Gross, J. (1971), *J. Lab. Clin. Med.* **78**, 275–288.
55. Carmel, R. (1975), *New. Engl. J. Med.* **292**, 282.
56. Howard, W. A., Bayomi, A., Natarajan, E., Aziza, M. A., El-Ahmady, O., Grissom, C. B., and West, F. G. (1997), *Bioconj. Chem.* **8**, 498–502.
57. Berridge, M. V., Tan, A. S., McCoy, D. K., and Wang, R. (1996), *Biochimica* **4**, 14.
58. Mitchell, A. M., master's thesis, University of Utah, 1997.
59. Wan, S., Parrish, J. A., Anderson, R. R., and Madden, M. (1981), *Photochem. Photobiol.* **34**, 679–681.
60. Wilksch, P. A., Jacka, F., and Blake, A. J. (1984), in *Porphyrin Localization and Treatment of Tumors* (Doiron, D. R., and Gomer, C. J., eds.), pp 149–161, Alan R. Liss, New York.
61. Brown, K. L., Cheng, S., Zubkowski, J. D., and Valente, E. J. (1996), *Inorg. Chem.* **35**, 3442–3446.

62. Suslick, K. S. (1990), *Science* **247**, 1439–1445.
63. Makino, K., Mossaba, M. M., and Riesz, P. (1983), *J. Phys. Chem.* **87**, 1369–1377.
64. Kondo, T., Krishna, C. M., and Riesz, P. (1990), *Free Rad. Res. Commun.* **10**, 27–35.
65. Makino, K., Mossaba, M. M., and Riesz, P. (1983), *Radiation Res.* **96**, 416–421.
66. Henglein, A. (1987), *Ultrasonics*. **25**, 6–16.
67. Kondo, T., Krishna, C. M., and Riesz, P. (1988), *Int. J. Radiat. Biol.* **5**, 331–342.
68. Tatterson, K. G. (1997), *Biophotonics International*, May/June, 34.
69. Bednarski, M. D., Lee, J. W., Callstrom, M. R., and Li, K. C. P. (1997), *Radiology* **204**, 263–268.
70. McKee, J. R., Christman, C. L., O'Brien, W. D., and Wang, S. Y. (1977), *Biochemistry* **16**, 4651–4654.
71. Flynn, H. G. (1982), *J. Acous. Soc. Am.* **72**, 1926.
72. Zuckier, L. S. and Chervu, L. T. (1984), *J. Nucl. Med.* **25**, 1032–1039.

16

INTRINSIC FACTOR, HAPTOCORRIN, AND THEIR RECEPTORS

DAVID H. ALPERS

Gastroenterology Division
Washington University School of Medicine
St. Louis, MO 63110

GREGORY J. RUSSELL-JONES

Cl-Biotech Australia Pty Ltd.
PO Box 20
Roseville, NSW, 2069 Australia

Chemistry and Biochemistry of B_{12}. Edited by Ruma Banerjee.
ISBN 0-471-25390-1.

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Carmel et al
Journal/Book Title:	Arch. Biochem. Biophys
Article Title:	
Volume (Issue):	184(1)
Pages:	135-140
Year of Publication:	1977
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASA	Date	

9184106 BIOSIS NO.: 199497192476

Hepatic cobalamin deficiency induced by hydroxycobalamin (c-lactam) treatment in rats is associated with decreased mitochondrial mRNA contents and accumulation of polycistronic mitochondrial RNAs.

AUTHOR: Leeds Frederic S; Brass Eric P(a)
AUTHOR ADDRESS: (a)Case Western Reserve Univ., Dep. Med., Div. Clinical Pharmacol., 10900 Euclid Ave., Cleveland, O**USA

JOURNAL: Journal of Biological Chemistry 269 (6):p3947-3951 1994
ISSN: 0021-9258
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Treatment of rats with hydroxycobalamin(c-lactam) (HCCL), a **cobalamin antagonist**, results in both increased hepatic mitochondrial content and the development of defects in mitochondrial ubiquinol:cytochrome c oxidoreductase and cytochrome c oxidase. The present study was designed to evaluate changes in hepatic mitochondrial RNA contents in response to HCCL treatment in rats. After 2 weeks of HCCL treatment, hepatic contents of the mature mitochondrial mRNAs (expressed normalized to 28 S rRNA) encoding subunit H of cytochrome c oxidase (CO II), subunit 1 of NADH dehydrogenase (ND1), and cytochrome b were reduced to values 40-60% of those observed in RNA from control liver tissue. In addition, HCCL induced a pronounced accumulation of high molecular weight RNA species which hybridized to mitochondrial probes and represented polycistronic RNA sequences. The polycistronic RNAs were products of the heavy strand of the mitochondrial genome, and major species demonstrated hybridization patterns consistent with identifications corresponding to the 12-16 S rRNA, 12-16 S-ND1, 16 S-ND1, and CO II-ATP synthase subunit 6 regions of the mitochondrial genome. Maximal expression of the polycistronic mitochondrial RNA was observed after 2 weeks of HCCL treatment. Thus, HCCL treatment interferes with mitochondrial RNA processing and decreases the content of mature mitochondrial mRNAs. Altered expression of the mitochondrial genome may be responsible for the decreased electron transport chain activity known to result from HCCL administration.

ıplete

)THER

s | 2nd

Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

8901601 BIOSIS NO.: 199396053102
Effects of vitamin B-12 analogues with alternations in the side chains of the corrin ring on urinary methylmalonate excretion in vitamin B-12-deficient rats.
AUTHOR: Saido Hisako; Watanabe Fumio; Tamura Yoshiyuki; Manai Yasuo; Nakano Yoshihisa(a)
AUTHOR ADDRESS: (a)Dep. Agric. Chem., Univ. Osaka Prefecture, Sakai, Osaka 593**Japan
JOURNAL: Bioscience Biotechnology and Biochemistry 57 (4):p607-610 1993
ISSN: 0916-8451
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: To study how much the side chains of the corrin ring of vitamin B-12 are involved in the physiological roles of the vitamin, five vitamin B-12 analogues (cyanocobalamin-b-monocarboxylate, cyanocobalamin-d-monocarboxylate, cyanocobalamin-e-monocarboxylate, cyano-13-epicobalamin, and cyanocobalamin(c-lactam)) with alternations in the side chains were synthesized chemically and then administered orally and intravenously to vitamin B-12-deficient rats. Male rats fed a vitamin B-12-deficient diet for 11 wk developed a severe vitamin B-12 deficiency with a high urinary methylmalonate excretion (223.8 +- 136.2 mu-mol/d) and -97% (1.2 +- 0.7 ng/g tissue) lower hepatic vitamin B-12 content. Oral and intravenous administration of cyanocobalamin-b-,-d-, and -e-monocarboxylates and cyano-13-epicobalamin could not improve the severe vitamin B-12-deficient status of the rats, indicating that the b-, d-, and e-propionamide side chains of the corrin ring of vitamin B-12 are important in the absorption, transport, and function of the vitamin in rats. Urinary methylmalonate excretion of the rats that were intravenously administered cyanocobalamin(clactam) increased twice as much as those of the other analogue-supplemented rats, suggesting that cyanocobalamin(c-lactam) act as a powerful **Cbl-antagonist**. The results also indicate that mammalian cells do not contain a system for synthesizing complete vitamin B-12 from these analogues.

Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date	Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name: Patricia Duffy | Org or A.U.: 1645 | Phone: 305-7555

Serial Number: 09/654,116 | Req. Date: AS NB | Date:

nplete

02732162 BIOSIS NO.: 000068042761
POSSIBILITY OF INCREASING THE ANTI NEOPLASTIC ACTION OF A FOLIC-ACID **ANTAGONIST** BY METHYL **COBALAMIN** ANALOGS
AUTHOR: SOF'INA Z P; MYASISHCHEVA N V; ARSENYAN F G; YURKEVICH A M
AUTHOR ADDRESS: ONCOL. SCI. CENT., ACAD. MED. SCI. USSR, MOSCOW, USSR.
JOURNAL: VESTN AKAD MED NAUK SSSR (1). 1979. 72-78. 1979
FULL JOURNAL NAME: Vestnik Akademii Meditsinskikh Nauk Sssr
CODEN: VAMNA
RECORD TYPE: Abstract
LANGUAGE: RUSSIAN

ABSTRACT: The effect of methylcobalamine and its analog (difluorochloromethylcobalamine and methylcobalamine chloropalladate) on the growth of transplantable tumors in mice [adenocarcinoma of the mammary gland (Ca-755), carcinoma of the uterine cervix (CUC-5) and carcinoma of the intestine (ACATOL)] was studied. The activity of the cobalamine coenzyme analogs was investigated when used alone or combined with inhibitors of dihydrofolate reductase and methionine synthetase. The results of the experiments indicate a stimulating effect of methylcobalamine on the growth of transplantable solid tumors in the animals. The antitumor activity of the methylcobalamine analog studied was higher in combined application with methotrexate. The most effective inhibition of tumor growth and longest survival of animals were achieved in combined application of methylcobalamine with methotrexate and methionine synthetase inhibitor, depending on the scheme of administration.

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

00295489 BIOSIS NO.: 000050110489
INDEX OF INHIBITION OF VITAMIN B-12 BY AN **ANTAGONIST COBALAMIN** MONO CARBOXYLIC-ACID ESCHERICHIA-COLI
AUTHOR: SHIMON A
JOURNAL: MIKROBIOLOGIYA 38 (2). 211-215. 1969. 1969
FULL JOURNAL NAME: Mikrobiologiya
CODEN: MIKBA
RECORD TYPE: Citation

Publication:	
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date	Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name: Patricia Duffy | Org or A.U.: 1645 | Phone: 305-7555

Serial Number: 09/654,116 | Req. Date: ASAP | Date:

lete

110147313 CA: 110(17)147313c JOURNAL
Effect of cobalamin inactivation on folate metabolism of leukemic cells
AUTHOR(S): Ermens, Anton A. M.; Kroes, Alois C. M.; Schoester, Martijn; Van Lom, Kirsten; Lindemans, Jan; Abels, Johan
LOCATION: Inst. Hematol., Erasmus Univ., Rotterdam, Neth.
JOURNAL: Leuk. Res. DATE: 1988 VOLUME: 12 NUMBER: 11-12 PAGES: 905-10
CODEN: LEREDD ISSN: 0145-2126 LANGUAGE: English
SECTION:
CA201006 Pharmacology
IDENTIFIERS: nitrous oxide cobalamin folate metab leukemia, neoplasm inhibitor folate metab nitrous oxide
DESCRIPTORS:
Neoplasm inhibitors,leukemia...
cobalamin antagonist nitrous oxide enhancement of, in humans, folate metab. alteration in
CAS REGISTRY NUMBERS:
10024-97-2 biological studies, cobalamin inactivation by, folate metab. of human leukemic cells response to
59-30-3 biological studies, metab. of, by human leukemic cells, cobalamin inactivation by nitrous oxide effect on
51-21-8 52-52-8 59-05-2 folate metab. of human leukemic cells alteration by cobalamin inactivation by nitrous oxide and, neoplasm inhibition in relation to
365-07-1 formation of, by human leukemic cells, cobalamin inactivation by nitrous oxide effect on
13408-78-1 inactivation of, by nitrous oxide, folate metab. of human leukemic cells response to

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name: Patricia Duffy | Org or A.U.: 1645 | Phone: 305-7555

Serial Number: 09/654,114 | Req. Date: ASAP | Date:

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

71010641 CA: 71(3)10641q JOURNAL
Inhibition of vitamin B12 by an antagonist, cobalamin monocarboxylic acid
AUTHOR(S): Simon, Agoston
LOCATION: Zavod. Farm. Khim. Prod., Budapest, Hung.
JOURNAL: Mikrobiologiya DATE: 1969 VOLUME: 38 NUMBER: 2 PAGES: 211-15
CODEN: MIKBA5 LANGUAGE: Russian
SECTION:
CA808000 Microbial Biochemistry
IDENTIFIERS: inhibitors vitamin B12, antagonists vitamin B12, cobalamin monocarboxylic acid
DESCRIPTORS:
Cobinic acid-pentamide,cyanide hydroxide, dihydrogen phosphate (ester), inner salt, 3'-ester with 5,6-dimethyl-1-.alpha.-D-ribofuranosylbenzimidazole...
vitamin B12 formation inhibition by
CAS REGISTRY NUMBERS:
68-19-9 biological studies, formation of, cobalamin monocarboxylic acid
inhibition of

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

67097883 CA: 67(21)97883j JOURNAL
Character of antagonism of cobalamin monocarboxylic acid toward some analogs of cobalamin
AUTHOR(S): Simon, Agoston
LOCATION: Fabrik Chem. Pharm. Prod. Chinoin, Budapest, Hung.
JOURNAL: Zentralbl. Bakteriol., Parasitenkd., Infektionskr. Hyg., Abt. 2: Allg., Landwirtsch. Tech. Mikrobiol. DATE: 1967 VOLUME: 121 NUMBER: 2
PAGES: 199-205 CODEN: ZBPIA9 LANGUAGE: German
SECTION:
CA808000 Microbial Biochemistry
IDENTIFIERS: VITAMIN B12 ANTAGONISTS, COBALAMINS MONOCARBOXYLIC ACID, CYANOCOBALAMIN ANTAGONISTS
DESCRIPTORS:

Remarks:

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Carmel etc.
Journal/Book Title:	Blood
Article Title:	
Volume (Issue):	49(6).
Pages:	~~950~~ 987 - 1000
Year of Publication:	1977
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Quadros et.
Journal/Book Title:	Blood
Article Title:	
Volume (Issue):	86 (10).
Pages:	p 489
Year of Publication:	1995 (Nov 15)
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	McKean et al.
Journal/Book Title:	Leukemia & Lymphoma
Article Title:	
Volume (Issue):	30 (1-2).
Pages:	101 - 109.
Year of Publication:	1998
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Marcoullis et al.
Journal/Book Title:	Br. Journal. Haematol.
Article Title:	
Volume (Issue):	43 (1).
Pages:	15-26.
Year of Publication:	1979
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Quadros etal.
Journal/Book Title:	Blood etal.
Article Title:	
Volume (Issue):	10 (suppl. 1).
Pages:	p 125A
Year of Publication:	1995
Publisher:	
Remarks:	
	Continue December 1-5 1995.

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Quadros et al.
Journal/Book Title:	Biochemical and Biophysical Research Communications
Article Title:	
Volume (Issue):	222 (1)
Pages:	149 - 154
Year of Publication:	1996.
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date	Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Herbert Victor
Journal/Book Title:	ACS Symp. Ser
Article Title:	
Volume (Issue):	277 (Xenobiot Metab: Nutr. Eff.)
Pages:	31-6
Year of Publication:	1985
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Miasishcheva et al
Journal/Book Title:	Voprosy meditsinskoi khimii
Article Title:	
Volume (Issue):	~~25~~ 23(5)
Pages:	622-9
Year of Publication:	1977 (Sept-Oct)
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Shimizu etal
Journal/Book Title:	Oncology
Article Title:	
Volume (Issue):	44(3)
Pages:	169-173
Year of Publication:	1987
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Nishizawa etal.
Journal/Book Title:	International journal for vitamin and nutrition research.
Article Title:	
Volume (Issue):	67(3)
Pages:	~~1640~~. 1641-70
Year of Publication:	1997
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Takahata et al.
Journal/Book Title:	J. Nutr. Sci Vitaminol.
Article Title:	
Volume (Issue):	41(5)
Pages:	515-526
Year of Publication:	1995
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date	Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Kroes ene.
Journal/Book Title:	Cancer Chemotherapy and Pharmacology
Article Title:	
Volume (Issue):	17/2
Pages:	114-120
Year of Publication:	1984
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	~~May~~ Myasishcheva ene.
Journal/Book Title:	VOPR. MED. KHIM
Article Title:	
Volume (Issue):	23(5)
Pages:	622-629.
Year of Publication:	1977
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	69/654, 114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Gaus etal.
Journal/Book Title:	Proc. Soc. Expr. Biol. Med.
Article Title:	
Volume (Issue):	149(2) : ~~388~~
Pages:	384-388.
Year of Publication:	1975
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,116	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	Hogenkamp et al (authors)/(Eds) Banerjee R.
Journal/Book Title:	Chemistry & Biochemistry of B12
Article Title:	
Volume (Issue):	
Pages:	p. 385-410
Year of Publication:	1999.
Publisher:	John Wiley & Sons - NY
Remarks:	

BOOK

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

INTER LIBRARY LOAN REQUEST FORM

Borrower's Name	Patricia Duffy	Org or A.U.	1645	Phone	305-7555
Serial Number	09/654,114	Req. Date	ASAP	Date	

Please Attach Copy of Abstract, Citation, or bibliography, If Available. Please Provide Complete Citation. Only One Request Per Form

Author/ Editor:	~~Pant~~ Pathare et al
Journal/Book Title:	Bioconjugate Chemistry
Article Title:	
Volume (Issue):	8(2)
Pages:	161-172
Year of Publication:	1997
Publisher:	
Remarks:	

STIC Use Only Accession Number: _____

Library Action	LC		NAL		NIH		NLM		NBS		PTO		OTHER	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
Local Attempts														
Date														
Initials														
Results														
Examiner Called														
Page Count														
Money Spent														

Provided By: Source and Date

Ordered From: Source and Date

Remarks/Comments

1st & 2nd dnotes time taken to a library

O/N - Under NLM means Overnight Service

FX - means Faxed to us

in the cellular uptake of vitamin B-12. The expression of megalin in absorptive epithelia in the kidney and other tissues including yolk sac and placenta suggests a role of the receptor in vitamin B-12 homeostasis and fetal vitamin B-12 supply.

8/7/6 (Item 6 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2002 BIOSIS. All rts. reserv.

10379877 BIOSIS NO.: 199699001022
Characterization of monoclonal %%%antibodies%%% to epitopes of human %%%transcobalamin%%% II.
AUTHOR: Quadros Edward V(a); Rothenberg Sheldon P; McLoughlin Patricia
AUTHOR ADDRESS: (a)Dep. Med., Div. Hematol./Oncol., SUNY-Health Sci. Cent., Brooklyn, NY 11203**USA
JOURNAL: Biochemical and Biophysical Research Communications 222 (1):p149-154 %%%1996%%%
ISSN: 0006-291X
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Cellular uptake of cobalamin (Cbl) is mediated by %%%transcobalamin%%% II (TCII), a Cbl binding protein in the plasma. The TCII-Cbl complex binds to a cell surface receptor and is internalized by endocytosis. We have generated monoclonal %%%antibodies%%% (mAbs) to human TCII that can be distinguished into three functional types on the basis of interaction with three different regions of the protein. Type 1: Receptor blocking. This mAb binds holo-TCII and inhibits the cellular uptake of Cbl. Type 2: Cbl blocking. This mAb binds apo-TCII at or near the Cbl binding domain and inhibits the formation of holo-TCII. Type 3: Precipitating. This mAb binds both holo-TCII and apo-TCII but does not interfere with Chi binding. Whereas type 1 and type 2 mAb, following incubation with TCII-(57Co)Cbl or apo-TCII, respectively, inhibit the uptake of radio-labeled Cbl by K562 cells, type 3 mAb has no such activity with either form of TCII. These properties of type 1 and type 2 mAb that inhibit the cellular uptake of Cbl, may serve to induce rapid Cbl deficiency and provide a model to study the effect of selective Cbl depletion on cell division and differentiation as well as on the pathways dependent on the two Cbl cofactors, methyl-Cbl and 5'-deoxyadenosyl-Cbl.

8/7/7 (Item 7 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2002 BIOSIS. All rts. reserv.

10245115 BIOSIS NO.: 199698700033
In vitro and in vivo inactivation of %%%transcobalamin%%% II receptor by its antiserum.
AUTHOR: Bose Santanu; Komorowski Richard; Seetharam Shakuntla; Gilfix Brian ; Rosenblatt David S; Seetharam Bellur(a)
AUTHOR ADDRESS: (a)MACC Res. Cent., Room 6061, Medical College Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 5**USA
JOURNAL: Journal of Biological Chemistry 271 (8):p4195-4200 %%%1996%%%
ISSN: 0021-9258
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

ABSTRACT: Rabbits injected with pure human placental %%%transcobalamin%%% II-receptor (TC II-R) failed to thrive with no apparent tissue or organ damage, but a 2-fold elevation of the metabolites, homocysteine, methylmalonic acid, and the ligand, %%%transcobalamin%%% II, in their plasma. Exogenously added %%%transcobalamin%%% II-(57Co)cyanocobalamin bound very poorly (2-5%) to the affected rabbit liver, kidney, and intestinal total or intestinal basolateral membrane extracts relative to the binding by membrane extracts from normal rabbit tissues. The activity was restored to normal values following a wash of affected rabbit tissue membranes with pH 3 buffer containing 200 mM potassium thiocyanate. Immunoblot analysis of normal and affected rabbit kidney and liver total membranes revealed similar amounts of 124-kDa TC II-R dimer protein. The neutralized and dialyzed extract from the affected rabbit membranes inhibited the binding of the ligand to pure TC II-R and the harvested affected rabbit serum inhibited the uptake of TC II-(57Co)cobalamin (Cbl) from the basolateral side of human intestinal epithelial (Caco-2) cells and decreased the utilization of (57Co)Cbl as coenzymes by the Cbl-dependent enzymes. The loss of exogenously added ligand binding or the binding of 125I-protein A occurred with the intestinal basolateral, but not the apical membranes. Based on these results, we suggest that circulatory %%%antibodies%%% to TC II-R cause its in vivo functional inactivation, suppress Cbl uptake by multiple tissues, and thus cause severe Cbl deficiency and the noted failure to thrive.

8/7/8 (Item 8 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2002 BIOSIS. All rts. reserv.

10165403 BIOSIS NO.: 199698620321
Use of monoclonal %%%antibodies%%% against %%%transcobalamin%%% II to inhibit cellular vitamin B12 uptake.
AUTHOR: McLean G R(a); Williams M J; Quadros E V; Schradern J W; Ziltener H J
AUTHOR ADDRESS: (a)Biomedical Res. Centre, Univ. British Columbia, Vancouver, BC**Canada
JOURNAL: Blood 86 (10 SUPPL. 1):p126A %%%1995%%%
CONFERENCE/MEETING: 37th Annual Meeting of the American Society of Hematology Seattle, Washington, USA December 1-5, 1995
ISSN: 0006-4971
RECORD TYPE: Citation
LANGUAGE: English

8/7/9 (Item 9 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2002 BIOSIS. All rts. reserv.

10165402 BIOSIS NO.: 199698620320
Epitope specific monoclonal %%%antibodies%%% (mAbs) to human %%%transcobalamin%%% II (TCII) can induce apoptosis by inhibiting the cellular uptake of cobalamin (Chl).
AUTHOR: Quadros Edward V; McLoughlin Patricia; Rothenberg Sheldon P; Morgan A Charles; Shikorska-Walker Marianna; Walker Roy
AUTHOR ADDRESS: Div. Hematology/Oncology, SUNY-Health Sci. Center, Brooklyn, NY**USA
JOURNAL: Blood 86 (10 SUPPL. 1):p125A %%%1995%%%
CONFERENCE/MEETING: 37th Annual Meeting of the American Society of Hematology Seattle, Washington, USA December 1-5, 1995
ISSN: 0006-4971
RECORD TYPE: Citation
LANGUAGE: English

8/7/10 (Item 10 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2002 BIOSIS. All rts. reserv.

09996655 BIOSIS NO.: 199598451573
The Rat Intrinsic Factor Gene: Its 5'-Upstream Region and Chief Cell-Specific Transcription.
AUTHOR: Maeda Masatomo(a); Asahara Seikyo; Nishi Tsuyoshi; Mushiake Sotaro; Oka Toshihiko; Shimada Shoichi; Chiba Tsutomu; Tohyama Masaya; Futai Masamitsu
AUTHOR ADDRESS: (a)Lab. Biochem., Fac. Pharm. Sci., Osaka Univ., 1-6-Yamada-oka, Suita, Osaka 565**Japan
JOURNAL: Journal of Biochemistry (Tokyo) 117 (6):p1305-1311 %%%1995%%%
ISSN: 0021-924X
DOCUMENT TYPE: Article

aspects]
%%%Vitamin%%% %%%B12%%% als Regulativ und Methotrexat als
%%%Antagonist%%% im Folsaure-Stoffwechsel. Pathophysiologische und
klinische Aspekte.
Sauer H
Fortschritte der Medizin (GERMANY, EAST) Apr 21 1983, 101 (15) p705-10, ISSN 0015-8178 Journal Code: 2984763R
Document type: Journal Article ; English Abstract
Languages: GERMAN
Main Citation Owner: NLM
Record type: Completed
Biochemical investigations show a decreased bioavailability of 5-methyl-tetrahydrofolic acid in %%%vitamin%%% %%%B12%%% deficient human
cell cultures and bone marrow cells. Tetrahydrofolic acid cannot be liberated from its storage form. This so-called methyl-folate-trap results in a functional folic acid deficiency which is the pathogenetic principle of the defect in the cell %%%proliferation%%% in patients with %%%vitamin%%% %%%B12%%% deficiency. This knowledge of biochemical
mechanisms leads to the identification of rare disorders in the metabolism of %%%vitamin%%% %%%B12%%% and folic acid. After methotrexate treatment a
rescue effect with its antidote Leucovorin can only be achieved, if the ratio antidote: methotrexate is at least 10:1. This ratio is important in cell cultures as well as in bone marrow cells in vivo. The results lead to a formula for the calculation of the optimal dosis to reach a secure rescue for individual patients after high-dose methotrexate treatment. This makes the high-dose methotrexate regimen a treatment modality for malignant tumors without any side effects.
Record Date Created: 19830715

5/7/40 (Item 14 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

03901350 82167942 PMID: 6461666
Effects of methyl-%%%B12%%% on the in vitro immune functions of human T
lymphocytes.
Sakane T; Takada S; Kotani H; Tsunematsu T
Journal of clinical immunology (UNITED STATES) Apr 1982, 2 (2) p101-9, ISSN 0271-9142 Journal Code: 8102137
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Studies were performed using an in vitro assay system to determine whether or not methyl-%%%B12%%% could affect human T-cell function. When T
cells were stimulated with phytohemagglutinin and allogeneic B cells, methyl-%%%B12%%% did not enhance T-cell %%%proliferation%%%. In contrast,
remarkable enhancing effects of methyl-%%%B12%%% on the %%%proliferative%%%
response to concanavalin A (Con A) and autologous B cells at suboptimal concentrations were observed, ranging from 0.1 to 10 micrograms/ml. Concentrations of methyl-%%%B12%%% sufficient to enhance cellular %%%proliferation%%% were able to enhance the activity of helper T cells for
immunoglobulin synthesis of B cells by pokeweed mitogen. Furthermore, the presence of methyl-%%%B12%%% significantly potentiated the induction of
suppressor cells in Con A-activated cultures. These results suggest that methyl-%%%B12%%% could modulate lymphocyte function through augmenting
regulatory T-cell activities.
Record Date Created: 19820624

5/7/41 (Item 15 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

03640330 81195859 PMID: 6262576
Modulation of EGF binding and action by succinylated concanavalin A in fibroblast cell cultures.
Ballmer K; Burger M M
Journal of supramolecular structure (UNITED STATES) 1980, 14 (2) p209-14, ISSN 0091-7419 Journal Code: 0330464
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
The role of the binding of succinylated concanavalin A to tissue culture cells in influencing epidermal growth factor (EGF)-mediated cell %%%proliferation%%% has been studied. Succinylated concanavalin A dramatically reduces the stimulation of 3T6 cells by EGF in Dulbecco's modified Eagle's medium (DME) containing insulin and %%%vitamin%%%
%%%B12%%% as additional growth factors, but no serum. Furthermore, binding
studies using 125I-labeled EGF have shown that the binding of EGF to the cell surface is reduced upon addition of succinylated concanavalin A.
Record Date Created: 19810709

5/7/42 (Item 16 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

03122425 79189368 PMID: 376110
Nutrients, vitamins and minerals as therapy.
Bertino J R
Cancer (UNITED STATES) May 1979, 43 (5 Suppl) p2137-42, ISSN
0008-543X Journal Code: 0374236
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Nutrients as therapy for patients with %%%cancer%%% are important as
adjunctive therapy, i.e., adequate nutrition may be important for the success of whatever form of therapy is administered. Diets deficient in certain amino acids have some selectivity when tested against experimental tumors propagated in vivo. Such diets have had limited clinical trial and have been characterized by poor patient acceptance. Enzymes that produce
deficiencies of certain amino acids, e.g., asparaginase, glutaminase, methioninase appear to offer a more reasonable approach to development of
selective amino acid deficiencies in man. Trace metals in excessive amounts may be toxic or carcinogenic to the host. Two heavy metal salts, Cis-diamine dichloroplatinum and gallium nitrate, have recently been shown to have anti-neoplastic effects in man. There is no conclusive evidence that vitamins, administered in large doses, have significant antineoplastic effects although large doses of %%%vitamin%%% A, %%%vitamin%%% C, and
%%%vitamin%%% %%%B12%%% have been used for this purpose. In contrast,
certain %%%vitamin%%% %%%analogs%%% such as folate antimetabolites can
cause tumor regression and are useful clinical treatment. An enzyme, carboxypeptidase G1, by splitting naturally occurring folates, may also have promise as a method of producing enzymic folate deficiency. (53 Refs.)
Record Date Created: 19790829

5/7/43 (Item 17 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

02759163 78076357 PMID: 339529
[Effect of methylcobalamin and fluoralkylcobalamins on E. coli 113/3 cell growth and on a primary human embryonic fibroblast culture]
Vliianie metilkobalamina i ftoralkilkobalaminov na rost kletok E. coli 113/3 i pervichnuiu kul'turu embrional'nykh fibroblastov cheloveka.
Miasishcheva N V; Golenko O D; Kuznetsova L E; Raushenbakh M O; Rudakova
I P
Voprosy meditsinskoi khimii (USSR) Sep-Oct 1977, 23 (5) p622-9,

ISSN 0042-8809 Journal Code: 0416601
Document type: Journal Article ; English Abstract
Languages: RUSSIAN
Main Citation Owner: NLM
Record type: Completed
Comparative analysis of the functional activity of several fluoralkylcobalamines was carried out using E. coli 113/3 strain deficient in %%%vitamin%%% %%%B12%%% and methionine. Difluoro chlor methylcobalamine
(CF2Cl-Cbl) exhibited the most distinct inhibitory effect on growth of bacterial cells in the medium with cobalamine. Effect of methylcobalamine and CF2Cl-Cbl on the %%%proliferative%%% activity of human embryonal
fibroblasts was studied in media of various composition. The %%%proliferative%%% activity of fibroblasts was distinctly increased in the
medium with methylcobalamine at various periods of cultivation; the fraction of 3H-thimidine labelled cells and the mitotic index were increased. The distinct decrease in amount of cells, synthesizing DNA, and in their mitotic activity was observed in medium with CF2Cl-Cbl. The data obtained suggest that difluorochlor methylcobalamine affects the cell %%%proliferation%%% as the %%%antagonist%%% of methylcobalamine in
experiments with bacterial cells and the primary culture of human embryonal fibroblasts.
Record Date Created: 19780223

5/7/44 (Item 18 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

02436620 77019051 PMID: 9787
%%%B12%%% -- dependent methionine synthetase as a potential target for
%%%cancer%%% chemotherapy.
Huennekens F M; DiGirolamo P M; Fujii K; Jacobsen D W; Vitols K S
Advances in enzyme regulation (ENGLAND) 1976, 14 p187-205, ISSN
0065-2571 Journal Code: 0044263
Document type: Journal Article; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
(51 Refs.)
Record Date Created: 19761121

5/7/45 (Item 1 from file: 370)
DIALOG(R)File 370:Science
(c) 1999 AAAS. All rts. reserv.

00510428 (USE 9 FOR FULLTEXT)
CHEMISTRY: Race for Molecular Summits
Service, Robert F.
Science Vol. 285 No. 5425 pp. 184
Publication Date: 07/09/1999 (990709) Publication Year: 1999
Document Type: Journal ISSN: 0036-8075
Language: English
Word Count: 2565

5/7/46 (Item 2 from file: 370)
DIALOG(R)File 370:Science
(c) 1999 AAAS. All rts. reserv.

00509101 (USE 9 FOR FULLTEXT)
Convergence of Transforming Growth Factor- (beta) and %%%Vitamin%%% D
Signaling Pathways on SMAD Transcriptional Coactivators
Yanagisawa, Junn; Yanagi, Yasuo; Masuhiro, Yoshikazu; Suzawa, Miyuki; Watanabe, Michiko; Kashiwagi, Kouji; Toriyabe, Takeshi; Kawabata, Masahiro; Miyazono, Kohei; Kato, Shigeaki<CRF RID="C1">
Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. CREST, Japan Science and Technology, 4-1-8 Honcho, Kawaguchi, Saitama 332, Japan. Department of Biochemistry, The Cancer Institute, Tokyo, Japanese Foundation for Cancer
Research (JFCR), and Research for the Future Program, Japan Society for the Promotion of Science, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170-8455, Japan.
Science Vol. 283 5406 pp. 1317
Publication Date: 2-26-1999 (990226) Publication Year: 1999
Document Type: Journal ISSN: 0036-8075
Language: English
Section Heading: REPORTS
Word Count: 2405

Abstract: Cell %%%proliferation%%% and differentiation are regulated by growth regulatory factors such as transforming growth factor- (beta) (TGF-
(beta)) and the liphophilic hormone %%%vitamin%%% D. TGF- (beta) causes activation of SMAD proteins acting as coactivators or transcription factors in the nucleus. %%%Vitamin%%% D controls transcription of target genes through the %%%vitamin%%% D receptor (VDR). Smad3, one of the SMAD proteins
downstream in the TGF- (beta) signaling pathway, was found in mammalian cells to act as a coactivator specific for ligand-induced transactivation of VDR by forming a complex with a member of the steroid receptor coactivator-1 protein family in the nucleus. Thus, Smad3 may mediate cross-talk between %%%vitamin%%% D and TGF- (beta) signaling pathways
References and Notes:

1. Mangelsdorf, D. J., et.al. Cell, 83 1995, 835 ;
2. Norman, A. W., Roth, J., Orchi, L., Endocr. Rev., 3 1982, 331 DeLuca, H. F., Adv. Exp. Med. Biol., 196 1986, 361 Walters, M. R., Endocr. Rev., 13 1992, 719 Hausller, M. R., et.al. J. Bone Miner. Res., 13 1998, 325 ;
3. Onate, S. A., et.al. Science, 270 1995, 1354 Kalkhoven, E., et.al. EMBO J., 17 1998, 232 Chen, H., et.al. Cell, 90 1997, 569 Torchia, J., et.al. Nature, 387 1997, 677 Horwitz, K. B., et.al. Mol. Endocrinol., 10 1996, 1167 Wurtz, J.-M., et.al. Nature Struct. Biol., 3 1996, 87 ;
4. Kamei, Y., et.al. Cell, 85 1997, 403 Chakravarti, D., et.al. Nature, 383 1996, 99 Kawasaki, H., et.al. ibid., 393 1998, 2845. Stern, P. H., Ed. by Feldman, D., Glorieux, F. H., Pike, J. W., %%%Vitamin%%% D, 1997, 343345 Academic Press, New York, , Takeshita,
A., et.al. J. Biol. Chem., 273 1998, 14738 ;
6. Yoshizawa, T., et.al. Nature Genet., 16 1997, 391 ;
7. Kato, S., et.al. Science, 270 1995, 1491 Takeyama, K., et.al. ibid., 277 1997, 1827 ;
8. Ebihara, K., et.al. Mol. Cell. Biol., 16 1996, 3393 ;
9. Kato, S., et.al. ibid., 15 1995, 5858 ;
10. COS-1 cells were maintained in Dulbecco's modified Eagle's medium without phenol red, supplemented with fetal bovine serum (5%) treated with dextran-coated charcoal. The cells were transfected at 40 to 50% confluency in 10-cm petri dishes with a total of 20 (mu) g of the indicated plasmids using calcium phosphate. All assays were done in the presence of 3 (mu) g of pCH110 (Pharmacia), a (beta) -galactosidase expression vector, as an internal control to normalize for variations in transfection efficiency. Cognate ligands were added to the medium 1 hour after transfection and at each exchange of medium. After 24-hour incubation with the calcium phosphate-precipitated DNA, the cells were washed with fresh medium and incubated for an additional 24 hours. Cell extracts were prepared by freezing and thawing and were assayed for CAT activity after normalization for (beta) -galactosidase activity as described (B9) . ;
11. The mammalian expression vector pcDNA3 (Invitrogen) was used for the expressions of Smad and SRC-1 proteins. Constitutively active and catalytically inactive forms of T (beta) R-I, BMPR-IA, and BMPR-IB were as described (%%%B12%%%) . Full-length VDR and VDR mutants were inserted
into the mammalian expression vector pSG5 (pSG5-VDR). DEF domains of VDR
were inserted into the pM vector (Clontech) [GAL4-VDR(DEF)] and full-length Smad2 and Smad3 were inserted into pVP (Clontech) (VP16-Smad2
and VP16-Smad3). ;
12. Imamura, T., et.al. Nature, 389 1997, 622 ;
13. J. Yanagisawa et al., unpublished data. ;
14. Massague, J., Cell, 85 1996, 947 Heldin, C.-H., Miyazono, K., ten Dijke, P., Nature, 390 1997, 465 Howe, J. R., et.al. Science, 280 1998, 1086 ;

frequency as a function of time, correlations among multiple markers of genetic damage, and influence on damage indices of nutritional variables, including blood levels of folate, %%%B12%%% and antioxidant vitamins. Among
different individuals, the range of values was 10-fold or more in the erythrocyte micronucleus, glycophorin A, plasma ascorbate and urinary 8-hydroxydeoxyguanosine (oxo8dG) assays, was approximately 6-fold in the
lymphocyte micronucleus assay, and was 2-fold in the lymphocyte sister chromatid exchange (SCE) assay. Red blood cell folate and plasma folate, %%%B12%%% and alpha-tocopherol values varied by up to 10-fold among
individuals. Micronucleus frequencies in erythrocytes and peripheral blood lymphocytes ranged from < 0.3 to 16.9/1000 in mature red blood cells, < 1 to 33/1000 in reticulocytes, and 2.5 to 15/1000 in binucleate lymphocytes. Frequencies of glycophorin A variant erythrocytes ranged from 5.6 to 77.3 x 10(6) N/0 cells and 3.2 to 16.2 x 10(6) N/N cells, and oxo8dG excretion varied from 32 to 397 pmol/kg/day. Although a wide range of values was observed in each genetic endpoint, the extreme values for various endpoints of genetic damage were not observed in the same individuals. The frequency of micronucleated erythrocytes varied over time within individuals and indicated that individuals with the highest levels of damage exhibit greater variability than those with lower levels. In some subjects, frequencies of micronucleated erythrocytes changed dramatically over an interval of 2-3 years: four subjects with initial micronucleated reticulocyte frequencies of 20.4, 5.9, 6.4 and 33/1000 changed to 2.5, 20.5, 18.5 and 12/1000, respectively. Among more than 150 individuals we have studied, including the 64 individuals studied by Everson et al. [(1988) J. Natl. %%%Cancer%%% Inst., 80, 525-529] and Smith et al. [(1990)
%%%Cancer%%% Res., 50, 5049-5054], the seven individuals with the highest
observed frequencies of micronucleated erythrocytes all had exceptionally low values of plasma folate, red cell folate, or plasma %%%B12%%%, suggesting that folate and %%%B12%%% status are the major determinants of
the types of damage that lead to spontaneous micronucleus formation in erythrocytic cells.
Record Date Created: 19970805

5/7/33 (Item 7 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09459226 97346559 PMID: 9202976
Effects of methylcobalamin on the %%%proliferation%%% of androgen-sensitive or estrogen-sensitive malignant cells in culture and in vivo.
Nishizawa Y; Yamamoto T; Terada N; Fushiki S; Matsumoto K; Nishizawa Y
Department of Pathology, Osaka Medical Center for Cancer, Japan.
International journal for vitamin and nutrition research. Internationale Zeitschrift fur Vitamin- und Ernahrungsforschung. Journal international de vitaminologie et de nutrition (SWITZERLAND) 1997, 67 (3) p164-70, ISSN 0300-9831 Journal Code: 1273304
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Methylcobalamin is one of the coenzymatically active cobalamin derivates and required for the activity of the cytoplasmic enzyme methionine synthetase catalyzing the methylation of homocysteine into methionine. The effect of methylcobalamin on the %%%proliferation%%% of malignant cells has
been examined. Methylcobalamin inhibited the %%%proliferation%%% of
androgen-sensitive SC-3 cells (a cloned cell line from Shionogi mouse mammary tumor, SC115) in culture at the concentration of 100-300 micrograms/ml. An inhibitory activity of methylcobalamin on the %%%proliferation%%% was also observed in other cell lines (estrogen-sensitive B-1F cells from mouse Leydig cell tumor and MCF-7 cells from human mammary tumor) at the concentration of 500 micrograms/ml.
Moreover, large doses of methylcobalamin injected intraperitoneally (100 mg/kg body weight/day) were non-toxic and suppressed the tumor growth of
SC115 and B-1F cells in mice fed a %%%vitamin%%% %%%B12%%% deficient diet.
These results show that methylcobalamin inhibits the %%%proliferation%%% of
malignant cells in culture and in vivo and propose the possibility of methylcobalamin as a candidate of potentially useful agents for the treatment for some malignant tumors.
Record Date Created: 19970916

5/7/34 (Item 8 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09016228 96375064 PMID: 8781445
Nitric oxide interactions with cobalamins: biochemical and functional consequences.
Brouwer M; Chamulitrat W; Ferruzzi G; Sauls D L; Weinberg J B
Duke University Marine Biomedical Center, Beaufort, NC, USA
Blood (UNITED STATES) Sep 1 1996, 88 (5) p1857-64, ISSN 0006-4971
Journal Code: 7603509
Contract/Grant No.: AR-39162; AR; NIAMS
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Nitric oxide (NO) is a paramagnetic gas that has been implicated in a wide range of biologic functions. The common pathway to evoke the functional response frequently involves the formation of an iron-nitrosyl complex in a target (heme) protein. In this study, we report on the interactions between NO and cobalt-containing %%%vitamin%%% %%%B12%%%
derivatives. Absorption spectroscopy showed that of the four Co(III) derivatives (cyanocobalamin [CN-Cbl], aquocobalamin [H2O-Cbl], adenosylcobalamin [Ado-Cbl], and methylcobalamin [MeCbl]), only the H2O-Cbl
combined with NO. In addition, electron paramagnetic resonance spectroscopy
of H2O-Cbl preparations showed the presence of a small amount of Cob-(II)alamin that was capable of combining with NO. The Co(III)-NO complex was very stable, but could transfer its NO moiety to hemoglobin (Hb). The transfer was accompanied by a reduction of the Co(III) to Co(II), indicating that NO+ (nitrosonium) was the leaving group. In accordance with this, the NO did not combine with the Hb Fe(II)-heme, but most likely with the Hb cysteine-thiolate. Similarly, the Co(III)-NO complex was capable of transferring its NO to glutathione. Ado-Cbl and Me-Cbl were susceptible to photolysis, but CN-Cbl and H2O-Cbl were not. The homolytic cleavage of the
Co(III)-Ado or Co(III)-Me bond resulted in the reduction of the metal. When
photolysis was performed in the presence of NO, formation of NO-Co(II) was
observed. Co(II)-nitrosyl oxidized slowly to form Co(III)-nitrosyl. The capability of aquocobalamin to combine with NO had functional consequences.
We found that nitrosylcobalamin had diminished ability to serve as a cofactor for the enzyme methionine synthase, and that aquocobalamin could
quench NO-mediated inhibition of cell %%%proliferation%%%. Our in vitro
studies therefore suggest that interactions between NO and cobalamins may
have important consequences in vivo.
Record Date Created: 19961016

5/7/35 (Item 9 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

08934564 96295494 PMID: 8702395
Relevance of the biosynthesis of coenzyme Q10 and of the four bases of DNA as a rationale for the molecular causes of %%%cancer%%% and a therapy.
Folkers K
Institute for Biomedical Research, University of Texas at Austin 78712, USA.
Biochemical and biophysical research communications (UNITED STATES)

decreasing high blood pressure, preventing colon %%%cancer%%% and kidney
stone formation. It is advantageous that milk contains three times more potassium than sodium. The antioxidant milk selenium decreases the risk of arteriosclerosis and %%%cancer%%% and also slows the ageing process. The
protective effects of different milk vitamins, e.g. %%%vitamin%%% A, beta
-carotene, %%%vitamin%%% E and %%%vitamin%%% C are well known. A recent
discovery is the role of folate-binding proteins, %%%vitamin%%% B6 and %%%B12%%% and folic acid of milk in the prevention of hyperhomocisteinemia-causing arteriosclerosis and thrombus formation. Recent research emphasises the importance of milk in its natural state, its contribution to the maintenance of the pH balance of the organism, and the fact that milk acts as a protective agent against the prions of BSE-disease.

5/7/9 (Item 1 from file: 73)
DIALOG(R)File 73:EMBASE
(c) 2002 Elsevier Science B.V. All rts. reserv.

03138491 EMBASE No: 1986161068
Enhanced therapeutic effect of methotrexate in experimental rat leukemia after inactivation of cobalamin (%%%vitamin%%% %%%B12%%%) by nitrous oxide
Kroes A.C.M.; Lindemans J.; Schoester M.; Abels J.
Institute of Hematology, Erasmus University, 3000 DR Rotterdam Netherlands
Cancer Chemotherapy and Pharmacology (CANCER CHEMOTHER. PHARMACOL.) (
Germany) 1986, 17/2 (114-120)
CODEN: CCPHD
DOCUMENT TYPE: Journal
LANGUAGE: ENGLISH

Exposure to nitrous oxide interferes selectively with the coenzyme function of %%%vitamin%%% %%%B12%%% and causes inactivation of methionine
synthetase, with subsequent impairment of folate metabolism and reduction of cellular %%%proliferation%%%. In a rat leukemia model (BNML) we investigated the combined administration of nitrous oxide, inactivating %%%vitamin%%% %%%B12%%%, and methotrexate (MTX), a folate %%%antagonist%%%
inhibiting the enzyme dihydrofolate reductase. Through different mechanisms, both agents decrease the availability of tetrahydrofolate, and subsequently of other reduced folates, with increased impairment of folate-dependent synthesis of thymidylate. Effects on leukemic growth and on hematological values in rats demonstrated enhancement of the therapeutic
effect of MTX by exposure to nitrous oxide. With several treatment schedules, the results of combined treatment were seen to be better than additive when compared with the effects of single agents. In particular, pretreatment of leukemic rats with nitrous oxide for 3 days before administration of MTX appeared effective. With higher doses of MTX, concomitant exposure to nitrous oxide even resulted in toxic effects. These findings were in accordance with the results of some metabolic studies performed in leukemic rats. De novo synthesis of thymidylate in leukemic cells, when studied by means of the deoxyuridine suppression test, showed the most severe disturbance with combined treatment consisting in MTX (0.5
mg/kg) and nitrous oxide pretreatment for 3 days. Intracellular levels of folate and dTTP were lowest with 2 and 3 days' pretreatment before MTX, respectively. It is concluded that this interaction of nitrous oxide and MTX can result in enhanced metabolic and therapeutic effects of low doses of MTX. Inactivation of %%%vitamin%%% %%%B12%%% appears to be a potentially
useful addition in %%%cancer%%% chemotherapy.

5/7/10 (Item 1 from file: 77)
DIALOG(R)File 77:Conference Papers Index
(c) 2002 Cambridge Sci Abs. All rts. reserv.

4609978
Supplier Accession Number: 01-06514 V29N05
Targeting of taxol analogs to cancer cells with vitamin B12
Bagnato, J.D.; West, F.G.; Grissom, C.B.
37th National Organic Symposium 0005671 Bozeman, MT (USA) 10-14
Jun 2001
Organic Division of the American Chemical Society
Montana State University, c/o Organic Chemistry Symposium, 280 Strand
Union, Bozeman, MT 59717, USA; phone: 406-994-3333; fax: 406-994-7070;
email: conferenceservices@montana.edu. Poster Paper No. 43
Languages: ENGLISH

5/7/11 (Item 1 from file: 94)
DIALOG(R)File 94:JICST-EPlus
(c)2002 Japan Science and Tech Corp(JST). All rts. reserv.

03184528 JICST ACCESSION NUMBER: 97A0548347 FILE SEGMENT: JICST-E
Occurrence of Cobalamin Coenzymes in the Photosynthetic Green Alga, Chlorella vulgaris.
WATANABE F (1); ABE K (1); TAKENAKA S (2); TAMURA Y (2); MARUYAMA I (3);
NAKANO Y (4)
(1) Kochi Women's Univ., Kochi, JPN; (2) Hagoromo-gakuen Coll., Osaka, JPN ; (3) Chlorella Ind. Co., Ltd., Fukuoka, JPN; (4)Osaka Prefecture Univ., Osaka, JPN
Biosci Biotechnol Biochem, 1997, VOL.61,NO.5, PAGE.896-897, FIG.1, TBL.1, REF.6
JOURNAL NUMBER: G0021ABU ISSN NO: 0916-8451 CODEN: BBBIE
UNIVERSAL DECIMAL CLASSIFICATION: 581.192
LANGUAGE: English COUNTRY OF PUBLICATION: Japan
DOCUMENT TYPE: Journal
ARTICLE TYPE: Short Communication
MEDIA TYPE: Printed Publication
ABSTRACT: To analyze cobalamin metabolism in photosynthetic green algae, the effects of cobalamin on growth of Chlorella vulgaris C-30 were studied and the algal cobalamin contents were assayed. Cobalamin significantly stimulated growth of the Chlorella cells, but biologically inactive cobalamin %%%analogues%%% did not. Chlorella grown in a cobalamin-free medium (control) contained cobalamin coenzymes, 5'-deoxyadenosylcobalamin (7.95.+-.0.31ng/g wet weight) and methylcobalamin (2.72.+-.0.45ng/g wet weight), of which the levels were increased significantly in cobalamin-supplemented cells. These results indicate that the alga has ability to take up exogenous cobalamin and synthesize the coenzyme forms. (author abst.)

5/7/12 (Item 2 from file: 94)
DIALOG(R)File 94:JICST-EPlus
(c)2002 Japan Science and Tech Corp(JST). All rts. reserv.

02588866 JICST ACCESSION NUMBER: 95A0977335 FILE SEGMENT: JICST-E
Synthesis, Properties and Microbiological Activity of Hydrophobic Derivatives of %%%Vitamin%%% %%%B12%%%.
TAKAHATA Y (1); NISHIZAWA A (1); YAMANISHI M (1); TORAYA T (1); KOJIMA I
(2)
(1) Okayama Univ., Okayama, JPN; (2) Nippon Oil Co., Ltd., Yokohama, JPN
J Nutr Sci Vitaminol, 1995, VOL.41,NO.5, PAGE.515-526, FIG.4, TBL.3, REF.18
JOURNAL NUMBER: F0733ABB ISSN NO: 0301-4800 CODEN: JNSVA
UNIVERSAL DECIMAL CLASSIFICATION: 577.164.1 579:577.3
LANGUAGE: English COUNTRY OF PUBLICATION: Japan
DOCUMENT TYPE: Journal
ARTICLE TYPE: Original paper
MEDIA TYPE: Printed Publication
ABSTRACT: Long chain alkylcobalamins and long chain acyl-cyanocobalamins, two types of hydrophobic derivatives of %%%vitamin%%% %%%B12%%%, were
synthesized. It was shown by TLC and determination of the partition coefficient between organic and aqueous phases that the hydrophobicity of alkylcobalamins and acyl-cyanocobalamins increased with the chain

length of the alkyl or acyl group introduced into cobalamin. Long chain alkylcobalamins were easily converted to aquacobalamin by photoirradiation, but the first-order rate constant of photolysis decreased with the length of an alkyl group. Long chain acyl-cyanocobalamins were gradually hydrolyzed to cyanocobalamin in neutral or alkaline solution with the pseudo-first order rate constant increasing with the pH of the solution. Stabilization of acyl-cyanocobalamins toward hydrolysis was achieved by introducing a methyl group into the .ALPHA.-position of an acyl group. All the long chain alkylcobalamins tested supported the growth of Escherichia coli 215, a cobalamin- or L-methionine-auxotroph, and Lactobacillus leichmannii, although their activity as cobalamin was at most 28% and 15% that of cyanocobalamin for E coli 215 and L. leichmannii, respectively. (author abst.)

5/7/13 (Item 3 from file: 94)
DIALOG(R)File 94:JICST-EPlus
(c)2002 Japan Science and Tech Corp(JST). All rts. reserv.

00935537 JICST ACCESSION NUMBER: 90A0241399 FILE SEGMENT: JICST-E
Isolation of extracellular cobalt-free corrinoid from Methanosarcina barkeri.
FUKUZAKI S (1); NISHIO N (1); NAGAI S (1)
(1) Hiroshima Univ., Higashi-Hiroshima, JPN
Agric Biol Chem, 1989, VOL.53,NO.9, PAGE.2455-2460, FIG.4, TBL.3, REF.19
JOURNAL NUMBER: G0021AAD ISSN NO: 0002-1369 CODEN: ABCHA
UNIVERSAL DECIMAL CLASSIFICATION: 579:577.3
LANGUAGE: English COUNTRY OF PUBLICATION: Japan
DOCUMENT TYPE: Journal
ARTICLE TYPE: Original paper
MEDIA TYPE: Printed Publication

5/7/14 (Item 1 from file: 98)
DIALOG(R)File 98:General Sci Abs/Full-Text
(c) 2002 The HW Wilson Co. All rts. reserv.

04674299 H.W. WILSON RECORD NUMBER: BGSA01174299 (THIS IS THE FULLTEXT)
The secular trends in male:female ratio at birth in postwar industrialized countries.
Jongbloet, Piet Hein
Zielhuis, Gerhard A; Groenewoud, Hans M. M
Environmental Health Perspectives (Environ Health Perspect) v. 109 no7 (July 2001) p. 749-52
LANGUAGE: English
COUNTRY OF PUBLICATION: United States
WORD COUNT: 4019

ABSTRACT: Finnish investigators (Vartiainen et al. Environmental Chemicals and Changes in Sex Ratio: Analysis Over 250 Years in Finland. Environ Health Perspect 107:813-815 (1999)) presented the sex ratio of all newborn babies from 1751 to 1997 in order to evaluate whether Finnish long-term data are compatible with the hypothesis that the decrease in the ratio of male to female births after World War I and World War II in industrial countries is caused by environmental factors. They found an increase in the proportion of males from 1751 to 1920, which was interrupted by peaks in male births during World War I and World War II and followed by a decrease thereafter, similar to the trends in many other countries. The turning point of male proportion, however, preceded the period of industrialization and introduction of pesticides and hormonal drugs. Thus, a causal association between these environmental exposures and this decrease is unlikely. In addition, none of the various family parameters (e.g., paternal age, maternal age, age difference in parents, birth order) could explain the historical time trends. Vartiainen et al. concluded that at present it is unknown how these historical trends could be mediated. The postwar secular decline of the male:female ratio at birth is not an isolated phenomenon and parallels the decline of perinatal morbidity and mortality, congenital anomalies, and various constitutional diseases. This parallelism indicates a common etiology and may be caused by reduction of conceptopathology, as a correlate to increasing socioeconomic development. An inverted dose response or the dose-response fallacy due to vanishing male conceptuses explains the low sex ratios before World War I and World War II in newborns from black parents and from the lowest socioeconomic classes. Reprinted by permission of the publisher.

TEXT:

Key words: neural tube defects, ovopathy, primary sex ratios, secondary sex ratios, socioeconomic patterning, tertiary sex ratios, vanishing male conceptuses. Environ Health Perspect 109:749-752 (2001). Online 13 July 2001
http://ehpnet1.niehs.nih.gov/docs/2001/109p749-752jongbloet/abstract.html

The decrease in the male:female sex ratio at birth, or the secondary sex ratio (SSR), generally found in post-World War II industrialized countries, such as the United States (1,2), Canada (1), Denmark (1,3,4), Germany (5), The Netherlands (1,4,5), and Finland (6), is a subject of continuing debate. The main hypothesis is that environmental chemical contaminants are responsible for this decrease. Fluctuation of the SSR has been promoted as a possible sentinel health indicator (4,7). However, current chemical pollution was virtually absent during the 18th and 19th centuries, which were characterized by a continuing increase of the SSR (1,2,5), as stressed by Vartiainen et al. (6). Environmental chemicals, therefore, do not explain the increasing SSRs before World War I and World War II or the decreasing ones afterward. In addition, environmental chemicals are unlikely to account for the increasing SSRs found among neonates born to black parents in the United States (3), nonmetropolitan areas of Italy (8), or low socioeconomic classes (9,10), compared to the simultaneously decreasing SSRs among those born to white parents in metropolitan areas or the upper classes.

In this paper we focus on the maturation of the oocyte and liquefaction of the cervical mucus in animal and human reproduction. We also offer a unifying concept that explains the fluctuations in sex determination at conception, that is, the primary sex ratio (PSR), and the shorter male life expectancy at every age from conception onward. Male-biased loss of pathologic conceptuses entails reversal of the secondary sex ratio (SSR) at birth; male-biased loss of children and adults affects the decline of the tertiary sex ratio during life, causing the increasing "gender gap."

Unifying hypothesis on sex determination. The intricate connections between either equal proportions of each sex and optimal conceptions at the core of the fertile window of the menstrual cycle, or between disproportionate rates of male-biased and pathologic conceptuses outside of this window are due to periovulatory hormone variation, which simultaneously modulates cervical liquefaction and oocyte maturation.

The cervical liquefaction plays a pivotal role in the migration of the spermatocytes (11), whereas developmental competence of the human oocyte is acquired during follicle formation and meiotic progression (12). Before midcycle, both liquefaction of the mucus plug and maturation of the oocyte are modulated by estrogens. Concurrence of both facilitates equal access and fertilization of optimally matured oocytes by X- and Y-bearing spermatocytes and full expression of the genetic potential resulting in good embryo quality.

In contrast, nonoptimal liquefaction and maturation due to hormonal disturbances occur at the very beginning and the end of the fertile window. Because the head, length, perimeter, and area of Y-bearing spermatozoa are significantly smaller than those of X-bearing spermatozoa, and their necks and tails are shorter (13), differential migration of the Y-bearing spermatozoa (14) and preferential fertilization of nonoptimally matured oocytes are likely. The pleiotropic nature of experimentally induced aging of the oocyte in animals before or after ovulation i.e. overripeness ovopathy (15-17) depends on molecular, biochemical, and physiologic processes in the oocyte, which encompasses both nuclear and cytoplasm constituents. The teratogenic results are impossibility of fertilization, improper implantation, prenatal loss, transitory retardation in the rate of development, and a spectrum of anomalies such as deficiencies in organogenesis or differentiation in various tissues and organ systems. Thus, ovopathy entails comorbidity of a broad spectrum of mutually interrelated conditions, and the teratogenic components apparently depend on degree and pleiotropic nature of overripeness ovopathy (18).

Dose-response sex ratio increase and reversal as a dose-response fallacy. This male-biased loss of conceptuses and individuals can evolve in sex ratio reversal, as shown by the overrepresentation of male blastocysts or births in rabbits after a short delay in fertilization and sex ratio reversal after a prolonged delay (19,20). Positive and, after having surpassed a certain threshold, negative dose-response gradients are

Naylor Stephen; Benson Linda M; Hardyman Timothy J; Thorson Linda M
AUTHOR ADDRESS: (a)Section of Nuclear Medicine, Mayo Clinic, 200 First St
SW, Rochester, MN, 55905**USA
JOURNAL: Mayo Clinic Proceedings 75 (6):p568-580 June, 2000
MEDIUM: print
ISSN: 0025-6196
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English
SUMMARY LANGUAGE: English

ABSTRACT: cntdot Objective: To study the biodistribution of a %%%vitamin%%% %%%B12%%% %%%analog%%%, indium In 111-labeled diethylenetriaminepentaacetate adenosylcobalamin (In 111 DAC), in patients recently diagnosed as having primary or recurrent malignancy. cntdot Patients and Methods: Thirty patients (14 women and 16 men) with radiographically or clinically diagnosed breast, lung, colon, sarcomatous, thyroid, or central nervous system malignancies were studied prior to definitive surgery or biopsy. A maximum of 650 muCi (2.2 mug) of In 111 DAC was administered intravenously. %%%Vitamin%%% %%%B12%%% and folate levels were determined prior to injection. Serum clearance and urinary and stool excretion of the tracer were measured. Images were routinely obtained at 0.5, 3 to 5, and 20 to 24 hours after injection. Biodistribution of In 111 DAC was determined by computer analysis of regions of interest. cntdot Results: Serum T1/2 clearance was 7 minutes. Average urinary and stool excretion of the injected dose over 24 hours was 26.1% and 0.4%, respectively. The greatest focal uptake of In 111 DAC occurred in the liver and spleen, followed by the nasal cavity and salivary and lacrimal glands. The average tumor uptake of the injected dose was 2% at 30 minutes and 1.5% at 24 hours. High-grade primary and metastatic breast, lung, colon, thyroid, and sarcomatous malignancies were all imaged at 3 to 5 hours after injection. Central nervous system tumors and advanced metastatic prostate %%%cancer%%% were best identified at 24 hours. Mammographically occult, palpable, and nonpalpable breast %%%cancers%%% were delineated by In 111 DAC. Low-grade malignancies as well as early skeletal metastatic disease were not effectively imaged by the %%%vitamin%%% %%%B12%%% tracer. Patients with elevated baseline %%%vitamin%%% %%%B12%%% or those concurrently taking corticosteroids appeared to have optimal visualization of their malignancies. cntdot Conclusion: %%%Vitamin%%% %%%B12%%% may be a useful vehicle for delivering diagnostic and therapeutic agents to various malignancies. Further evaluation of cobalamin %%%analogs%%% and their interaction with transport proteins and cellular receptors within malignant tissue and infection is warranted.

5/7/2 (Item 2 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2002 BIOSIS. All rts. reserv.

02615660 BIOSIS NO.: 000067003718
EFFECT OF METHYL COBALAMIN AND FLUOROALKYL COBALAMINS ON GROWTH OF ESCHERICHIA-COLI 113-3 CELLS AND ON THE PRIMARY CULTURE OF HUMAN EMBRYO FIBROBLASTS
AUTHOR: MYASISHCHEVA N V; GOLENKO O D; KUZNETSOVA L E; RAUSHENBAKH M O; RUDAKOVA I P; TACHKOVA E M; YURKEVICH A M
AUTHOR ADDRESS: ONCOL. SCI. CENT., ACAD. MED. SCI. USSR, MOSCOW, USSR.
JOURNAL: VOPR MED KHIM 23 (5). 1977 (RECD 1978) 622-629. 1977
FULL JOURNAL NAME: Voprosy Meditsinskoi Khimii
CODEN: VMDKA
RECORD TYPE: Abstract
LANGUAGE: RUSSIAN

ABSTRACT: Comparative analysis of the functional activity of several fluoroalkylcobalamins was carried out using E. coli 113/3 strain deficient in %%%vitamin%%% %%%B12%%% and methionine. Difluorochlormethylcobalamin (Cf2Cl-Cbl) exhibited the most distinct inhibitory effect on growth of bacterial cells in the medium with cobalamin. Effect of methylcobalamin and CF2Cl-Cbl on the %%%proliferative%%% activity of human embryonal fibroblasts was studied in media of various composition. The %%%proliferative%%% activity of fibroblasts was distinctly increased in the medium with methylcobalamin at various periods of cultivation; the fraction of 3H-thymidine labeled cells and the mitotic index were increased. The distinct distinct decrease in amount of cells, synthesizing DNA and in their mitotic activity was observed in medium with CF2Cl-Cbl. The data suggest that difluorochlormethylcobalamin affects the cell %%%proliferation%%% as the %%%antagonist%%% of methylcobalamin in experiments with bacterial cells and the primary culture of human embryonal fibroblasts.

5/7/3 (Item 1 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci
(c) 2002 Inst for Sci Info. All rts. reserv.

05422678 Genuine Article#: VY448 Number of References: 19
Title: NATURE KNOWS BEST - AN AMAZING REACTION CASCADE IS UNCOVERED BY DESIGN AND DISCOVERY
Author(s): HEATHCOCK CH
Corporate Source: UNIV CALIF BERKELEY,DEPT CHEM/BERKELEY//CA/94720
Journal: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, 1996, V93, N25 (DEC 10), P14323-14327
ISSN: 0027-8424
Language: ENGLISH Document Type: ARTICLE
Abstract: The Daphniphyllum alkaloids are a group of highly complex polycyclic alkaloids, Examination of the structures if several members of this family of natural products led to a hypothesis about their mode of biosynthesis (depicted in Scheme I), Based on this hypothetical biosynthetic pathway, a laboratory synthesis was designed that incorporated as a key transformation the novel one-pot transformation of dialdehyde 24 to pentacyclic unsaturated amine 25, This process turned out to be an exceptionally efficient way to construct the pentacyclic nucleus of the Daphniphyllum alkaloids, However, a purely fortuitous discovery, resulting from accidental use of methylamine rather than ammonia, led to a great improvement in the synthesis and suggests an even more attractive possible biosynthesis.

5/7/4 (Item 2 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci
(c) 2002 Inst for Sci Info. All rts. reserv.

03604843 Genuine Article#: PQ988 Number of References: 105
Title: THE LOCAL LYMPH-NODE ASSAY - DEVELOPMENTS AND APPLICATIONS
Author(s): KIMBER I; DEARMAN RJ; SCHOLES EW; BASKETTER DA
Corporate Source: ZENECA CENT TOXICOL LAB,ALDERLEY PK/MACCLESFIELD SK10 4TJ/CHESHIRE/ENGLAND/; UNILEVER ENVIRONM SAFETY LAB/SHARNBROOK MK44 1LQ/BEDS/ENGLAND/
Journal: TOXICOLOGY, 1994, V93, N1 (SEP 23), P13-31
ISSN: 0300-483X
Language: ENGLISH Document Type: ARTICLE
Abstract: The murine local lymph node assay is a predictive test method for the identification of contact allergens in which sensitizing activity is measured as a function of induced %%%proliferative%%% responses in lymph nodes draining the site of application. In this article the development and validation of the assay are described and comparisons with guinea pig predictive test methods discussed. In addition we examine the advantages and limitations of the method and consider new opportunities and applications of the assay in the context of the toxicological evaluation of sensitizing potential.

Int J Hematol, 1992, VOL.56,NO.2, PAGE.167-177, FIG.7, REF.28
JOURNAL NUMBER: F0888ABI ISSN NO: 0925-5710 CODEN: IJHEE
UNIVERSAL DECIMAL CLASSIFICATION: 616-006-09 577.164.1
LANGUAGE: English COUNTRY OF PUBLICATION: Japan
DOCUMENT TYPE: Journal
ARTICLE TYPE: Original paper
MEDIA TYPE: Printed Publication
ABSTRACT: Cobalamin-deficient P388D1 mouse leukemic cells were created by
propagation in a cyanocobalamin-freemedium in which the original fetal bovine serum was replaced by bovine serum albumin. These cobalamin-deficient cells gradually ceased to multiply when the medium contained 5-methyltetrahydrofolate. The growth of cells that had been cultured with this coenzyme was recovered following the addition of cyanocobalamin(CNCbl), at concentrations above 37pM. In contrast to the effect of CNCbl, cobinamide, and cobalamin %%%analogues%%% prepared from hydroxy cobalamin by reaction with ascorbic acid, did not have a growth-inducing effect on these cells, nor did these %%%analogues%%% inhibit CNCbl-dependent growth. %%%Transcobalamin%%% II-cobalamin complex had a remarkably stimulating effect on cell growth. The growth. inducing effect became apparent with a cobalamin concentration of only 0.37pM. This was about 1/100th the level of free cobalamin required for cell growth. However, no growth-inducing effect was seen at an R protein-bound cobalamin concentration of 37pM, indicating that once cobalamin has been bound to R protein, it loses its growth-promoting effect on these cells in culture. (author abst.)

3/7/36 (Item 1 from file: 144)
DIALOG(R)File 144:Pascal
(c) 2002 INIST/CNRS. All rts. reserv.

00929909 PASCAL No.: 76-0056396
METABOLISM OF CYANOCOBALAMIN BY L-1210 LEUKEMIC LYMPHOBLASTS
(LE METABOLISME DE LA CYANOCOBALAMINE PAR LES LYMPHOBLASTES DE LA LEUCEMIE L-1210)
GAMS R A; RYEL E M; MEYER L M
COMPR. CANCER CENT., BIRMINGHAM,ALA. 35294
Journal: PROC. SOC. EXPER. BIOL. MED., 1975, 149 (2) 384-388
Availability: CNRS-3031
No. of Refs.: 10 REF.
Document Type: P (SERIAL) ; A (ANALYTIC)
Country of Publication: USA
Language: ENGLISH
LES CELLULES DE L-1210 NE CONTIENNENT PAS ELLES-MEMES DE PROTEINE %%%ANALOGUE%%% A LA TRANSCOBALAMINE II (%%%TCII%%%) DANS LA PHASE SOLUBLE CYTOPLASMATIQUE. UNE PORTION DE LA VITAMINE %%%B12%%% LIEE A CETTE PROTEINE A ETE CONVERTIE EN FORMES ADENOSYL- ET METHYL- ET CES FORMES COENZYMATIQUES PEUT-ETRE TRANSFORMEES A L'EXTERIEUR DE LA CELLULE

3/7/37 (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09246625 97132938 PMID: 8978297
Antibodies to %%%transcobalamin%%% II block in vitro proliferation of leukemic cells.
McLean G R; Quadros E V; Rothenberg S P; Morgan A C; Schrader J W;
Ziltener H J
Biomedical Research Centre, University of British Columbia, Vancouver, Canada.
Blood (UNITED STATES) Jan 1 1997, 89 (1) p235-42, ISSN 0006-4971
Journal Code: 7603509
Contract/Grant No.: RO1-DK28561-14; DK; NIDDK
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed

The plasma protein %%%transcobalamin%%% II (%%%TCII%%%) binds and
delivers cobalamin (Cbl; %%%vitamin%%% %%%B12%%%) to all cells, which
internalize the %%%TCII%%% /Cbl complex by receptor-mediated endocytosis.
Congenital deficiency of %%%TCII%%% results in intracellular Cbl deficiency, one effect of which is to disrupt DNA synthesis, leading to megaloblastic anemia. We report here an in vitro culture system in which cell growth is dependent on delivery of Cbl to cells by %%%TCII%%%. Recombinant human holo-%%%TCII%%% was shown to support in dose-dependent
manner the growth of the human erythroleukemic cell line K562 and the murine lymphoma cell line BW5147. Free Cbl also supported cell growth; however, at 100- to 1,000-fold higher concentrations than those effective in the presence of apo-%%%TCII%%%. To determine if cellular depletion of
Cbl could be achieved by interfering with interactions between %%%TCII%%%
/Cbl and its cell-surface receptor, several monoclonal antibodies raised against human %%%TCII%%% were studied. Three antibodies, found to compete
for the same binding site on %%%TCII%%%, proved to be effective inhibitors
of %%%TCII%%% /Cbl-dependent cell growth. Our results suggest that
monoclonal anti-%%%TCII%%% antibodies that block the function of this
protein may prove useful in antitumor therapies.
Record Date Created: 19970127

3/7/38 (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

07987340 94125560 PMID: 8295340
%%%Vitamin%%% %%%B12%%% and %%%transcobalamin%%% in chronic
myeloproliferative disorders]
Iseki T
1st Department of Internal Medicine, School of Medicine, Chiba University.
Rinsho byori. The Japanese journal of clinical pathology (JAPAN) Dec 1993, 41 (12) p1310-21, ISSN 0047-1860 Journal Code: 2984781R
Document type: Journal Article ; English Abstract
Languages: JAPANESE
Main Citation Owner: NLM
Record type: Completed
Although %%%vitamin%%% %%%B12%%% is an essential coenzyme for DNA
synthesis, humans, like other mammals, are incapable of synthesizing it. The role of intrinsic factor (IF) in %%%B12%%% absorption is widely known,
but, in fact there exists a much more intricate and complex mechanism for the effective assimilation of this important trace element in humans. %%%B12%%% binding proteins play important roles in all stages of %%%vitamin%%% %%%B12%%% metabolism. They are involved not only in its
absorption, but also in its transport in serum, uptake to cells, storage in organs, enterohepatic circulation, and elimination of its %%%analogues%%%. Besides IF, well-known as a %%%vitamin%%% %%%B12%%% binding protein found
in gastric juice, there are other kinds of binding proteins found in human serum which are composed to %%%transcobalamin%%% (TC) I, II and III.
Elevation of the %%%vitamin%%% %%%B12%%% level in chronic myelogenous
leukemia was first reported in the 1950s. Since then, %%%B12%%% elevation
has been found to occur in other kinds of chronic myeloproliferative disorders (CMPDs) as well and to be caused by an increase of serum TC. In CMPDs, either TCI or TCIII increases, but, the degree of elevation and the type of TC involved differs for each disorder. This article describes the changes in TC of CMPD patients. With the induction of the developed radioimmunoassay for R-type %%%B12%%% binding protein, many cases have been

Title: EFFECT OF THE COBALT-N COORDINATION ON THE COBAMIDE RECOGNITION BY
THE HUMAN %%%VITAMIN%%%-%%%B12%%% BINDING-PROTEINS INTRINSIC-FACTOR,
%%%TRANSCOBALAMIN%%% AND HAPTOCORRIN
Author(s): STUPPERICH E; NEXO E
Corporate Source: UNIV ULM,ANGEW MIKROBIOL ABT,ALBERT EINSTEIN ALLEE
11/D-7900 ULM//FED REP GER/; CENT HOSP HILLEROD,DEPT CLIN CHEM/HILLEROD//DENMARK/
Journal: EUROPEAN JOURNAL OF BIOCHEMISTRY, 1991, V199, N2, P299-303
Language: ENGLISH Document Type: ARTICLE
Abstract: The binding of several corrinoids to the binding site of human intrinsic factor, %%%transcobalamin%%% or haptocorrin was investigated.
p-Cresolyl cobamide and 2-amino-%%%vitamin%%% B-12 are complete corrinoids, whose nucleotide at the lower face of the corrin ring is not coordinated to the cobalt. These corrinoids were greater-than-or-equal-to 10(3) times less efficiently recognized by intrinsic factor or %%%transcobalamin%%% than %%%vitamin%%% B-12, which
contains a Co-coordinated nucleotide. Pseudovitamin B-12, With a weak Co-N coordination bond, revealed only moderate affinity to intrinsic factor. From these findings it is concluded that the cobamide binding to intrinsic factor and %%%transcobalamin%%% is strongly affected by the Co-N coordination bonds of their lower cobalt nucleotide ligands. We suggest that the Co-N coordination bond positions the nucleotide at a critical distance to the corrin ring, which is recognized by the binding proteins.

Human haptocorrin, however, disclosed to distinctive selectivity regarding the different corrinoid structures. The protein bound all corrinoids with similar efficiency, independent of the strength of their Co-N coordinations, or the structures of their lower Co-alpha-ligands. Hence, the corrin ring, rather than a structural feature induced by the Co-N coordination, has to be considered responsible for the corrinoid binding to haptocorrin.

3/7/25 (Item 11 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci
(c) 2002 Inst for Sci Info. All rts. reserv.

00842735 Genuine Article#: FA890 Number of References: 43
Title: STUDIES ON THE %%%TRANSCOBALAMIN%%% RECEPTOR IN HOG KIDNEY
Author(s): YAMADA S; RIITTINEN L; MAJURI R; FUKUDA M; GRASBECK R
Corporate Source: MINERVA FDN,TUKHOLMANKATU 2/SF-00250 HELSINKI//FINLAND/;
MINERVA FDN,TUKHOLMANKATU 2/SF-00250 HELSINKI//FINLAND/; SAPPORO MED COLL,DEPT LAB DIAG/SAPPORO/HOKKAIDO060/JAPAN/
Journal: KIDNEY INTERNATIONAL, 1991, V39, N2, P289-294
Language: ENGLISH Document Type: ARTICLE
Abstract: The binding of the cobalamin-%%%transcobalamin%%% complex by its
solubilized receptor from hog kidney membrane was studied. The receptor bound the complex in a system containing bivalent cations, and the affinity was dependent on the NaCl concentration but not on temperature. The binding of cobalamin-%%%transcobalamin%%% to the receptor had an association constant of approximately 4.6 x 10(9) liter/mol and it was saturable and highly specific as %%%competition%%% by other proteins was not observed. The receptor had higher affinity for the cobalamin-%%%transcobalamin%%% complex (holo-TC) than for %%%transcobalamin%%% (apo-TC). Basic amino compounds known to interfere with tubular reabsorption of proteins did not inhibit the binding. Studies on subcellular fractions supported the view that the receptor was located on the brush border membrane of the kidney 2.

3/7/26 (Item 1 from file: 50)
DIALOG(R)File 50:CAB Abstracts
(c) 2002 CAB International. All rts. reserv.

03899650 CAB Accession Number: 20001412515
Diagnostic and therapeutic %%%analogues%%% of cobalamin.
Hogenkamp, H. P. C.; Collins, D. A.; Grissom, C. B.; West, F. G.
Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455, USA.
Chemistry and biochemistry of %%%B12%%%.
p.385-410
Publication Year: 1999
Editors: Banerjee, R.
Publisher: John Wiley and Sons -- New York, USA
ISBN: 0-471-25390-1
Language: English
Document Type: Miscellaneous
This chapter examines the diagnostic and therapeutic %%%analogues%%% of
%%%vitamin%%% %%%B12%%% . Section headings include: Transport of
cobalamins; Absorption of cobalamins; Modification of cobalamins; Diagnostic and therapeutic cobalamin %%%analogues%%%; Cobalt substitution;
Radioiodination of cobalamin; Cobalamin-chelator bioconjugates; Gadolinium-labelled cobalamin; Therapeutic uses of cobalamin %%%analogues%%% ; Targeted drug delivery; Depletion and deprivation of
cobalamin; Bioconjugate structure and recognition by %%%transcobalamin%%%;
and delivery of therapeutic agents. 72 ref.

3/7/27 (Item 2 from file: 50)
DIALOG(R)File 50:CAB Abstracts
(c) 2002 CAB International. All rts. reserv.

03899637 CAB Accession Number: 20001412502
Chemistry and biochemistry of %%%B12%%%.
Department of Biochemistry, University of Nebraska, Lincoln, NE, USA.
xxii + 921 pp.
Publication Year: 1999
Editors: Banerjee, R.
Publisher: John Wiley and Sons -- New York, USA
ISBN: 0-471-25390-1
Language: English
Document Type: Book
This book examines the chemistry, biochemistry and biology of %%%vitamin%%% %%%B12%%% . Individual chapters focus on: X-ray
crystallography; X-ray absorption spectroscopy; the roles of cobalt, corrin and protein; electron paramagnetic resonance of %%%vitamin%%%
%%%B12%%% -dependent enzymes; NMR spectroscopy; magnetic field dependence
of cobalamin photochemistry and enzymes; stereospecificity of the coenzyme
%%%B12%%%-catalysed rearrangements; %%%B12%%% electrochemistry and
organometallic electrochemical synthesis; %%%vitamin%%% %%%B12%%% and
nutrition; inborn errors of cobalamin metabolism; diagnostic and therapeutic %%%analogues%%% of cobalamin; intrinsic factor, haptocorrin
and their receptors; %%%transcobalamin%%% II; mammalian receptors and
%%%vitamin%%% %%%B12%%%-binding proteins; cobalamin transport in bacteria;
biosynthesis of %%%vitamin%%% %%%B12%%% in Pseudomanas denitrificans;
biosynthesis of the 5,6-dimethylbenzimidazole moeity of cobalamin and of the other bases found in natural corrinoids; regulation of adenosyl biosynthesis in Salmonella typhimurium; acetogenic corrinoid proteins; the role of corrinoids in methanogenesis; cobalamin-dependent methionine synthase; methylmalonyl-CoA mutase; ribonucleotide reductases; glutamate
mutase and 2-methyleneglutarate mutase; diol dehydratase and glycerol dehydratase; ethanolamine ammonia-lyase; aminomutases; isobutyryl-CoA mutase; and reductive dehalogenases. Many ref.

unnecessary. The binding capacity of TFS does not increase significantly with increasing concentrations of cyanocobalamin as does the binding capacity of intrinsic factor, normal serum or %%%transcobalamin%%% I. A single extract was prepared from each of 44 sera and measured for %%%vitamin%%% %%%B12%%% content simultaneously by the TFS assay and the conventional microbiologic method using Lactobacillus leichmannii. The values obtained with TFS were in each instance higher than those obtained by the microbiologic assay (P < 0.001).

3/7/15 (Item 1 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci

10495586 Genuine Article#: 533UR Number of References: 25
Title: Comparative analysis of cobalamin binding kinetics and ligand protection for intrinsic factor, %%%transcobalamin%%%, and haptocorrin
Author(s): Fedosov SN (REPRINT) ; Berglund L; Fedosova NU; Nexo E; Petersen TE
Corporate Source: Aarhus Univ,Prot Chem Lab, Dept Mol & Struct Biol,Sci Pk,Gustav Wieds Vej 10/DK-8000 Aarhus C//Denmark/ (REPRINT); Aarhus Univ,Prot Chem Lab, Dept Mol & Struct Biol,DK-8000 Aarhus C//Denmark/; Aarhus Univ,Dept Biophys,DK-8000 Aarhus//Denmark/; Aarhus Univ Hosp,AKH , Dept Clin Biochem,DK-8000 Aarhus C//Denmark/
Journal: JOURNAL OF BIOLOGICAL CHEMISTRY, 2002, V277, N12 (MAR 22), P 9989-9996
ISSN: 0021-9258 Publication date: 20020322
Publisher: AMER SOC BIOCHEMISTRY MOLECULAR BIOLOGY INC, 9650 ROCKVILLE PIKE, BETHESDA, MD 20814-3996 USA
Language: English Document Type: ARTICLE
Abstract: Changes in the absorbance spectrum of aquo-cobalamin (Cbl.OH2) revealed that its binding to %%%transcobalamin%%% (TC) is followed by slow conformational reorganization of the protein-ligand complex (Fedosov, S. N., Fedosova, N. U., Nexo, E., and Petersen, T. E. (2000) J. Biol. Chem. 275, 11791-11798). Two phases were also observed for TC when interacting with a Cbl-%%%analogue%%% cobinamide (Cbi), but not with other cobalamins. The slow phase had no relation to the ligand recognition, since both Cbl and Cbi bound rapidly and in one step to intrinsic factor (IF) and haptocorrin (HC), namely the proteins with different Cbl specificity. Spectral transformations observed for TC in the slow phase were similar to those upon histidine complexation with Cbl.OH2 and Cbi. In contrast to a closed structure of TC.Cbl.OH2, the %%%analogous%%% IF and HC complexes revealed accessibility of Cbl's upper face to the external reagents. The binders decreased sensitivity of adenosyl-Cbl (Cbl.Ado) to light in the range: free ligand, IF., HC., TC.Cbl.Ado. The spectrum of TC.Cbl.Ado differed from those of IF and HC and mimicked Cbl.Ado participating in catalysis. The above data suggest presence of a histidine-containing cap shielding the Cbl-binding site in TC. The cap coordinates to certain corrinoids and, possibly, produces an incapsulated Ado-radical when Cbl.Ado is bound.

3/7/16 (Item 2 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci

09415956 Genuine Article#: 402UN Number of References: 31
Title: %%%Analogues%%%, ageing and aberrant assimilation of %%%vitamin%%% %%%B12%%% in Alzheimer's disease
Author(s): McCaddon A (REPRINT) ; Hudson P; Abrahamsson L; Olofsson H; Regland B
Corporate Source: Gardden Rd Surgery,Rhosllanerchrugog/Wrexham/Wales/ (REPRINT); Gardden Rd Surgery,Rhosllanerchrugog/Wrexham/Wales/; Wrexham Maelor Hosp,Wrexham//Wales/; Uddevalla Hosp,Dept Clin Chem,Uddevalla//Sweden/; Uddevalla Hosp,Dept Neuropsychiat,Uddevalla//Sweden/; Sahlgrens Univ Hosp,Dept Neuropsychiat,Molndal//Sweden/
Journal: DEMENTIA AND GERIATRIC COGNITIVE DISORDERS, 2001, V12, N2 (MAR-APR), P133-137
ISSN: 1420-8008 Publication date: 20010300
Publisher: KARGER, ALLSCHWILERSTRASSE 10, CH-4009 BASEL, SWITZERLAND
Language: English Document Type: ARTICLE
Abstract: %%%Vitamin%%% %%%B12%%% assimilation might be disrupted in patients with Alzheimer's disease. We therefore measured %%%B12%%% carrier protein saturation and inactive %%%B12%%% '%%%analogues%%%' in patients compared with healthy elderly individuals in a prospective case-controlled survey, Twenty-three patients, aged 60 or over, with features compatible with DSM-IV criteria for primary degenerative dementia of the Alzheimer type were recruited together with 18 cognitively intact age-matched control subjects. Total %%%vitamin%%% %%%B12%%% (active corrinoids), holo- and apo-haptocorrin and %%%transcobalamin%%% were measured in serum. %%%B12%%% %%%analogues%%% (inactive corrinoids) were estimated from the difference between R-binder-determined corrinoids and an intrinsic factor based %%%B12%%% assay. Alzheimer patients had significantly lower active corrinoid than control subjects a nd the %%%analogue%%%/corrinoid ratio was significantly higher in the Alzheimer group. The inter-relationship between age, %%%analogues%%% and %%%transcobalamin%%% polarised patients into two distinct groups. Two disparate mechanisms might exist for the development of cerebral %%%B12%%% deficiency in Alzheimer's disease, although both imply a disruption of selective %%%B12%%% assimilation and %%%analogue%%% elimination in such patients. Copyright (C) 2001 S. Karger AG, Basel.

3/7/17 (Item 3 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci

05684860 Genuine Article#: WQ215 Number of References: 27
Title: Synthesis of cobalamin dimers using isophthalate cross-linking of corrin ring carboxylates and evaluation of their binding to %%%transcobalamin%%% II
Author(s): Pathare PM; Wilbur DS (REPRINT) ; Hamlin DK; Heusser S; Quadros EV; McLoughlin P; Morgan AC
Corporate Source: UNIV WASHINGTON,DEPT RADIAT ONCOL, 2121 N 35TH ST/SEATTLE//WA/98103 (REPRINT); UNIV WASHINGTON,DEPT RADIAT ONCOL/SEATTLE//WA/98195; SUNY HLTH SCI CTR,VA MED CTR/BROOKLYN//NY/11209; RECEPTAGEN CORP,/EDMONDS//WA/98020
Journal: BIOCONJUGATE CHEMISTRY, 1997, V8, N2 (MAR-APR), P161-172
ISSN: 1043-1802 Publication date: 19970300
Publisher: AMER CHEMICAL SOC, 1155 16TH ST, NW, WASHINGTON, DC 20036
Language: English Document Type: ARTICLE
Abstract: Several cobalamin (Cbl) dimers have been prepared for evaluation as potential antiproliferative agents in the treatment of AIDS-related lymphoma. The Cbl dimers were synthesized by cross-linking Cbl carboxylates, produced by acid hydrolysis of the b-, d-, and e-propionamide side chains of cyanocobalamin (CN-Cbl), through an isophthalate molecule. Linking molecules were used between the Cbl carboxylates and the isophthalate moiety. The linkers were incorporated to provide a distance between the two Cbl molecules such that the dimeric Cbls might bind two molecules of %%%transcobalamin%%% II (%%%TCII%%%), the Cbl transport protein in plasma. Initially, the linking moiety used was 1,12-diaminododecane, but the resulting dimers had low aqueous solubility. To improve the solubility of the dimers, 4,7,10-trioxa-1,13-tridecanediamine was employed as the linking moiety. This improved the water solubility of the dimers considerably, while retaining the distance between the Cbl molecules at 41-42 Angstrom (fully extended). To introduce additional substitution on Cbl dimers, 5-aminoisophthalic acid was used as the cross-linking reagent.

ABSTRACT: By starch gel electrophoresis and autoradiography 2 classes of %%%vitamin%%% %%%B12%%% binding proteins were detected in rabbit serum.
By %%%analogy%%% to the nomenclature used in man, the 2 classes of proteins were named %%%transcobalamin%%% I (TCI) and %%%transcobalamin%%% II (%%%TCII%%%). Fifteen %%%TCII%%% phenotypes were observed, and family data indicated that they are controlled by 5 allelic codominant genes. The possibility that the 5 genes arise from the action of at least 2 polymorphic and closely linked structural loci is discussed.

3/7/12 (Item 12 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2002 BIOSIS. All rts. reserv.

02384210 BIOSIS NO.: 000065041246
ABSORPTION PLASMA TRANSPORT AND CELLULAR RETENTION OF COBALAMIN %%%ANALOGS%%% IN THE RABBIT EVIDENCE FOR THE EXISTENCE OF MULTIPLE MECHANISMS THAT PREVENT THE ABSORPTION AND TISSUE DISSEMINATION OF NATURALLY OCCURRING COBALAMIN %%%ANALOGS%%%
AUTHOR: KOLHOUSE J F; ALLEN R H
AUTHOR ADDRESS: DIV. HEMATOL., UNIV. COLO. MED. CENT., DENVER, COLO. 80220, USA.
JOURNAL: J CLIN INVEST 60 (6). 1977 1381-1392. 1977
FULL JOURNAL NAME: Journal of Clinical Investigation
CODEN: JCINA
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: %%%Analogs%%% of cobalamin (Cbl; %%%vitamin%%% %%%B12%%%) are prevalent in nature as a result of bacterial synthesis and are of additional interest because of their potential use as antimetabolites and chemotherapeutic agents. Fourteen Cbl %%%analogs%%% containing 57Co were synthesized and their gastrointestinal absorption, plasma transport and cellular retention were compared to that of [58Co]Cbl in rabbits. Many of the Cbl %%%analogs%%% were bound with low affinity by intrinsic factor and none of these [57CO]Cbl %%%analogs%%% were taken up by the ileum or absorbed into the body in amounts comparable to that of [58Co]Cbl. The Cbl %%%analogs%%% that were bound by intrinsic factor with high affinity were taken up by the ileum but in many cases were retained there in significant amounts. Most of the Cbl %%%analogs%%% were bound by plasma %%%transcobalamin%%% II with high affinity and all of these %%%transcobalamin%%% II-[57Co]Cbl %%%analog%%% complexes were taken up by a variety of tissues in a manner that was indistinguishable from that of %%%transcobalamin%%% II-[58Co]Cbl. The few %%%analogs%%% that were bound by %%%transcobalamin%%% II with low affinity were taken up by tissues in lesser amounts and 20-70% of these %%%analogs%%% was rapidly excreted in the urine as occurs with native Cbl when it is present in plasma in unbound form. All of the Cbl %%%analogs%%% were bound by the granulocyte R-type Cbl-binding protein with high affinity and all of the R-type protein [57Co]Cbl %%%analog%%% complexes were cleared rapidly from plasma exclusively by hepatocytes as occurs with R-type protein-[58Co]Cbl. Some Cbl %%%analogs%%% were released back into the plasma and were disseminated among a variety of tissues via %%%transcobalamin%%% II as occurs with native Cbl. Other Cbl %%%analogs%%% were retained in the liver and eventually excreted in the feces and urine without accumulating in other tissues. Intrinsic factor and the ileum prevent certain Cbl %%%analogs%%% from entering the body and the granulocyte R-type protein and hepatocytes prevent the dissemination of certain Cbl %%%analogs%%% that may gain entry, such as during infections with Cbl %%%analog%%% -producing bacteria. The fact that %%%transcobalamin%%% II binds and transports a large number of Cbl %%%analogs%%% indicates that these protective mechanisms can be circumvented and supports the feasibility of using Cbl %%%analogs%%% as antimetabolites in vivo.

3/7/13 (Item 13 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2002 BIOSIS. All rts. reserv.

02373642 BIOSIS NO.: 000065030672
THE EFFECT OF RHODIUM AND COPPER %%%ANALOGS%%% OF COBALAMIN ON HUMAN CELLS IN-VITRO
AUTHOR: CARMEL R; KOPPENHAGEN V B
AUTHOR ADDRESS: UNIV. SOUTH. CALIF. SCH. MED., 2025 ZONAL AVE., LOS ANGELES, CALIF. 90033, USA.
JOURNAL: ARCH BIOCHEM BIOPHYS 184 (1). 1977 135-140. 1977
FULL JOURNAL NAME: Archives of Biochemistry and Biophysics
CODEN: ABBIA
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: Rh and Cu %%%analogs%%% of various cobalamins produce an anti- %%%vitamin%%% %%%B12%%% effect in bacteria, suggesting that a similar inhibitory activity could be demonstrated for human cells. The %%%analogs%%% competed effectively with cyanocobalamin for binding by human serum transcobalamins. Methylrhodibalamin and 5'-deoxyadenosylrhodibalamin also competed with cyanocobalamin for serum-mediated uptake by human blood cells and bone marrow cells, though the %%%competition%%% was relatively weak when compared to the effective %%%competition%%% for %%%transcobalamin%%% II binding. None of the %%%analogs%%% affected normoblastic bone marrow cells, using deoxyuridine suppression of [3H]thymidine incorporation into DNA as the index of %%%vitamin%%% %%%B12%%% sufficiency. Methylrhodibalamin actively corrected the abnormality in %%%vitamin%%% %%%B12%%%-deficient bone marrow. However, 5'-deoxyadenosylrhodibalamin worsened the %%%vitamin%%% %%%B12%%%-deficient behavior of megaloblastic bone marrow and inhibited its correction by %%%vitamin%%% %%%B12%%% and may even have advesely affected 1 of the 5 normoblastic marrows tested.

3/7/14 (Item 14 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2002 BIOSIS. All rts. reserv.

02176008 BIOSIS NO.: 000064018521
COMPARISON OF TOADFISH SERUM %%%COMPETITIVE%%% BINDING AND MICROBIOLOGIC ASSAYS OF %%%VITAMIN%%% B-12
AUTHOR: BUCHANAN J W; MCINTYRE P A; SCHEFFEL U; WAGNER H N JR
JOURNAL: J NUCL MED 18 (4). 1977 394-398. 1977
FULL JOURNAL NAME: Journal of Nuclear Medicine
CODEN: JNMEA
RECORD TYPE: Abstract

ABSTRACT: Toadfish serum (TFS) offers several advantages over other proteins as the binder in a %%%competitive%%%-binding assay for %%%vitamin%%% %%%B12%%%. It is unaffected by pH changes in the range 5.6-9.4 or by the addition of human serum albumin. Prolonged incubation with charcoal does not disrupt the TFS-cyanocobalamin bond, and the addition of albumin as a protein source in the standard tubes was proven

Jul
16 1996, 224 (2) p358-61, ISSN 0006-291X Journal Code: 0372516
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
In the human, coenzyme Q10 (%%%vitamin%%% Q10) is biosynthesized from
tyrosine through a cascade of eight aromatic precursors. These precursors indispensably require eight vitamins, which are tetrahydrobiopterin, vitamins B6, C, B2, %%%B12%%%, folic acid, niacin, and pantothenic acid as their coenzymes. Three of these eight vitamins (the coenzyme B6, and the coenzymes niacin and folic acid) are indispensable in the biosynthesis of the four bases (thymidine, guanine, adenine, and cytosine) of DNA. One or more of the three vitamins required for DNA are known to cause abnormal
pairing of the four bases, which can then result in mutations and the diversity of %%%cancer%%%. The coenzyme B6, required for the conversion of
tyrosine to p-hydroxybenzoic acid, is the first coenzyme required in the cascade of precursors. A deficiency of the coenzyme B6 can cause dysfunctions, prior to the formation of %%%vitamin%%% Q10, to DNA. Former
data on blood levels of Q10 and new data herein on blood levels of B6, measured as EDTA, in %%%cancer%%% patients established deficiencies of Q10
and B6 in %%%cancer%%%. This complete biochemistry relating to biosyntheses
of Q10 and the DNA bases is a rationale for the therapy of %%%cancer%%%
with Q10 and other entities in this biochemistry.
Record Date Created: 19960830

5/7/36 (Item 10 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05507196 87259376 PMID: 3601319
Experimental study of antitumor effect of methyl-%%%B12%%%.
Shimizu N; Hamazoe R; Kanayama H; Maeta M; Koga S
Oncology (SWITZERLAND) 1987, 44 (3) p169-73, ISSN 0030-2414 Journal Code: 0135054
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
We examined the antitumor effect of %%%vitamin%%% %%%B12%%% (methyl-
%%%B12%%%) using C3H/He, C57BL/6 and BALB/C mice for animals and MH134
hepatoma ascites cells, Lewis lung %%%cancer%%% cells and Ehrlich ascites
tumor cells for tumor cells. At 1.0-10 micrograms/ml, methyl-%%%B12%%%
enhanced PHA- and Con-A-induced lymphocyte blastoformation of C3H/He mice.
The growth of MH134 tumors on the backs of C3H/He mice were suppressed by
the 7-day administration of 50 or 100 micrograms/day i.p. and their survival was longer than that of untreated mice. However, methyl-%%%B12%%%
administration did not positively affect the survival of C3H/He mice that had been irradiated with 60Co 300 R on the day before tumor cell inoculation. The growth of Ehrlich ascites tumor cells inoculated into BALB/C mice was also reduced at 17 and 19 days after tumor inoculation by administration of methyl-%%%B12%%% 50 micrograms/day i.p. and the mice
survived longer than the untreated mice.
Record Date Created: 19870727

5/7/37 (Item 11 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

05361399 87114053 PMID: 2433545
Serum %%%vitamin%%% %%%B12%%% levels in patients with primary
hepatocellular carcinoma during treatment with CB3717.
Buamah P K; James O F; Skillen A W; Harris A L
Journal of surgical oncology (UNITED STATES) Feb 1987, 34 (2) p100-3
, ISSN 0022-4790 Journal Code: 0222643
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Elevated Serum %%%B12%%% levels were found at diagnosis in five of eleven
patients with primary hepatocellular carcinoma. During chemotherapy either with CB3717 or VP16 the serum %%%B12%%% rose dramatically, in one case
reaching levels ten times the upper limit of normal. However, the serum LD activities did not change in parallel with the serum %%%B12%%% levels suggesting that there was little necrosis of the tumour or the liver. With two out of five patients with other types of %%%cancer%%% the serum
%%%B12%%% levels also increased but less markedly. This data seems to
suggest that the serum %%%B12%%% level may not be as good a tumour marker
for hepatocellular carcinoma as has been suggested and indeed may be influenced by the chemotherapeutic agent.
Record Date Created: 19870319

5/7/38 (Item 12 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

04668367 85046998 PMID: 6498076
Synergistic growth inhibiting effect of nitrous oxide and cycloleucine in experimental rat leukaemia.
Kroes A C; Lindemans J; Abels J
British journal of cancer (ENGLAND) Dec 1984, 50 (6) p793-800, ISSN 0007-0920 Journal Code: 0370635
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: Completed
Nitrous oxide (N2O) inactivates the %%%vitamin%%% %%%B12%%%-dependent
enzyme methionine synthetase with subsequent impairment of folate metabolism and a reduction of cellular %%%proliferation%%%. Indications exist that this effect is antagonized by S-adenosylmethionine (SAM), and it was investigated whether combination with an inhibitor of SAM synthesis, cycloleucine, would result in increased inhibition of growth in rat leukaemia model (BNML). Leukaemic growth was compared in untreated rats, in
rats treated with either nitrous oxide/oxygen (1:1) or cycloleucine (50 mg kg-1 i.p.), and in rats receiving both agents. Combined treatment resulted in the strongest reduction of leukaemic infiltration in spleen and liver, and this reduction often was more than the added effects of single treatments. Peripheral leukocyte counts were also lowest after combined treatment. The deoxyuridine suppression test, measuring folate-dependent de
novo synthesis of thymidine, was more severely disturbed with combined treatment. Levels of %%%vitamin%%% %%%B12%%% in plasma were reduced in rats
receiving N2O, but an increase in plasma folate occurred in all treated rats. These results indicate that a reduction of SAM synthesis by cycloleucine can increase the disturbance of folate metabolism that is caused by nitrous oxide, with a potentiation of the effects on leukaemic growth.
Record Date Created: 19850117

5/7/39 (Item 13 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

04216788 83211490 PMID: 6602086
%%%Vitamin%%% %%%B12%%% as a regulator and methotrexate as an
%%%antagonist%%% of folic acid metabolism. Pathophysiologic and clinical

intact guinea pigs and nondissociable radioactivity was extracted 2-4.5 h later. The proportion of extracted 57Co eluting as free cobalamin increased to 39-46%, that eluting as intrinsic factor-cobalamin complex declined to 22-45%, and 9-34% now eluted as a macromolecule that reacted with antitranscobalamin II %%%antibody%%% but not antiintrinsic factor %%%antibody%%%. Extracted 35S radioactivity eluted in several peaks in addition to the intrinsic factor peak. After reversible attachment of intrinsic factor-cobalamin complex to its ileal surface receptor, an energy-dependent process prevents removal of the complex from the cell surface by EDTA or acid; cobalamin dissociates from intrinsic factor and, as suggested by previous workers, binds to a molecule antigenically similar to %%%transcobalamin%%% II; and intrinsic factor is slowly degraded and forms breakdown products that are detectable in ileal extracts.

8/7/25 (Item 25 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

03507250 BIOSIS NO.: 000073010330
IMMUNO BLASTIC LYMPH ADENOPATHY
AUTHOR: VAN DIJK Y M; HEYD Y; DOLLBERG L
AUTHOR ADDRESS: DEP. GERIATR., DAY HOSP., JERUSALEM.
JOURNAL: HAREFUAH 100 (1). 1981. 12-17, 55. %%%1981%%%
FULL JOURNAL NAME: Harefuah
CODEN: HAREA
RECORD TYPE: Abstract
LANGUAGE: HEBREW

ABSTRACT: Immunoblastic lymphadenopathy (ILD), first described in 1974, affects mainly lymph nodes and lymphoid organs and typically manifests itself by generalized lymphadenopathy, hepatosplenomegaly, fever, rash, hypergammaglobulinemia and Coomb's-positive hemolytic anemia. The diagnosis is made by finding the typical polymorphic picture of proliferating small vessels, immunoblasts and deposits of amorphous interstitial material in the affected nodes. Three cases of ILD are described. One patient survived for 2 yr after diagnosis, went into remission after a course of combined chemotherapy (COPP) [cyclophosphamide, oncovin, procarbazine and prednisone], relapsed after antibiotic treatment and died of sepsis after transformation of the disease into immunoblastic sarcoma. The other 2 patients died of sepsis a few months after diagnosis, although no malignant change was found in repeated biopsies. In one of them, monoclonal gammopathy (IgG.KAPPA.) was demonstrated. In this patient, antismooth muscle %%%antibody%%%, a positive latex test for rheumatoid factor, a low complement level, Coombs'-positive hemolytic anemia and reduced migration of lymphocyte surface receptors to concanavalin A were found. This phenomenon has been described as typical of lymphocytes of malignant lymphoma and chronic lymphatic leukemia. Of the level of %%%transcobalamin%%% II was high in 2 patients in whom it was tested. This phenomenon has also been described as occurring in lymphoproliferative diseases.

8/7/26 (Item 26 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

02957475 BIOSIS NO.: 000069065593
SPECIES SPECIFICITY IN THE IMMUNOLOGIC REACTIONS AND BIOLOGICAL FUNCTIONS
OF TRANS COBALAMIN II
AUTHOR: HAUS M; GREEN P D; HALL C A
AUTHOR ADDRESS: HEMATOL. RES, VETERANS ADM. MED. CENT., ALBANY, N.Y. 12208,
USA.
JOURNAL: PROC SOC EXP BIOL MED 162 (2). 1979. 295-298. %%%1979%%%
FULL JOURNAL NAME: Proceedings of the Society for Experimental Biology and Medicine
CODEN: PSEBA
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: Reactivity between antihuman %%%transcobalamin%%% (TC)II and the TC II from 8 species [baboon, dog, rabbit, guinea-pig, goat, horse, pig and chicken] of animals was evaluated by a radioimmunoassay for human TC II. None of the animal TC IIs reacted to the same degree as an equal amount of human TC II, although the TC II of the baboon expressed partial reactivity. The function of each TC II in promoting the uptake of Cbl (cobalamin) was evaluated by exposure to cultured human HeLa cells and human lymphocytes. There was considerable variation in potency with some TC II having no effect, while others, such as that of the baboon, were almost as active as human TC II. The same species of TC II expressed varied potency for promoting uptake by murine fibroblasts as well. %%%Antibody%%% against human TC II and the TC II mediated complete uptake of Cbl by human cells exhibit species specificity. The TC II-Cbl from some species was less effective than murine TC II-Cbl or the murine L-929 system, but some forms of TC II-Cbl were more effective.

8/7/27 (Item 27 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)

02936330 BIOSIS NO.: 000069044448
BLOCKING AND BINDING TYPE %%%ANTIBODIES%%% AGAINST ALL MAJOR VITAMIN B-12 BINDERS IN A PERNICIOUS ANEMIA SERUM
AUTHOR: MARCOULLIS G; PARMENTIER Y; NICOLAS J-P
AUTHOR ADDRESS: DIV. HAEMATOL. ONCOL., N.Y. MED. COLL., COHEN RES. BUILD., 5TH AVE. AT 106TH ST., NEW YORK, N.Y. 10029, USA.
JOURNAL: BR J HAEMATOL 43 (1). 1979. 15-26. %%%1979%%%
FULL JOURNAL NAME: British Journal of Haematology
CODEN: BJHEA
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

ABSTRACT: The simultaneous occurrence of blocking and binding %%%antibodies%%% to intrinsic factor (IF), %%%transcobalamin%%% (TC II, TC I) and other R type vitamin B12 proteins in the serum of a patient with treated pernicious anemia (PA) was reported for the 1st time. The dialyzed and purified immunoglobulin (Ig) G, but not the IgM, from a PA patient neutralized the total unsaturated vitamin B12 binding capacity (UB12BC) of human gastric juice, saliva and serum and also of rabbit serum, suggesting that the PA IgG contained blocking %%%antibodies%%% against IF, TC II, TC I and other R-binders. In addition, the PA IgG and IgM preparations contained binding %%%antibodies%%% since they could form macromolecular complexes with 57Co-B12 bound to IF, TC I or TC II so that each one of the latter was totally excluded from Sephadex G-200. The presence of the binding %%%antibodies%%% was further confirmed by the formation of radioactive precipitation lines on agarose with each of the vitamin B12-binders bound to 58Co-B12. The PA serum did not exhibit any measurable UB12BC after dialysis against 7.5 M guanidine-HCl followed by renaturation with phosphate buffer (pH 7.4). It formed TC I and TC II complexes with 57Co-B12 when the latter was added during the renaturation step, indicating that the serum contained circulating Ig-TC complexes. The blocking %%%antibodies%%% should be distinguished from the previously described binding %%%antibodies%%%. The blocking of the binding of vitamin B12 to TC resulted in lower serum vitamin B12 levels in the present case in contrast to the presence of binding %%%antibodies%%% where high serum vitamin B12 levels were reported. The binding %%%antibodies%%% form immunocomplexes with TC which can easily be detected because they can bind radioactive vitamin B12 while the corresponding immunocomplexes of blocking %%%antibodies%%% are hidden because they prevent the binding of the vitamin to TC.

CA802000 General Biochemistry
IDENTIFIERS: Stokes radius transcobalamins, radius vitamin B12 transcobalamins, antibodies transcobalamin radius
DESCRIPTORS:
Insulins,properties...
Stokes radius of
Vitamin B12,transcobalamin complexes...
Stokes radius of, lipoprotein-depleted blood plasma effect on
Transcobalamin I...
vitamin B12 complexes, Stokes radius of
Transcobalamin II...
vitamin B12 complexes, Stokes radius of, lipoprotein-depleted blood plasma effect on

8/7/104 (Item 5 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
(c) 2002 AMERICAN CHEMICAL SOCIETY. All rts. reserv.

69017513 CA: 69(5)17513s JOURNAL
Antibody to transcobalamin II in patients treated with long acting vitamin B12 preparations
AUTHOR(S): Olesen, Henrik; Hom, B. L.; Schwartz, M.
LOCATION: Bispebjerg Hosp., Copenhagen, Den.
JOURNAL: Scand. J. Haematol. DATE: 1968 VOLUME: 5 NUMBER: 1 PAGES:
5-16 CODEN: SJHAAQ LANGUAGE: English
SECTION:
CA812000 Mammalian Pathological Biochemistry
IDENTIFIERS: anemia transcobalamin, vitamin B12 anemia, antibodies transcobalamin
DESCRIPTORS:
Transcobalamin II...
antibodies to, after vitamin B12 prepn. treatment
Antibodies...
to transcobalamin II after vitamin B12 prepn. treatment
CAS REGISTRY NUMBERS:
68-19-9 biological studies, antibodies to transcobalamin II after treatment with

8/7/105 (Item 1 from file: 434)
DIALOG(R)File 434:SciSearch(R) Cited Ref Sci
(c) 1998 Inst for Sci Info. All rts. reserv.

06391740 Genuine Article#: AFU48 Number of References: 37
Title: RECEPTOR DISTRIBUTION AND THE ENDOTHELIAL UPTAKE OF %%%TRANSCOBALAMIN%%%-II IN LIVER-CELL SUSPENSIONS
Author(s): SODA R; TAVASSOLI M; JACOBSEN DW
Corporate Source: VET ADM MED CTR/JACKSON//MS/39216; UNIV MISSISSIPPI,MED
CTR,SCH MED/JACKSON//MS/39216; SCRIPPS CLIN & RES FDN,DEPT BASIC & CLIN
RES,DIV BIOCHEM/LA JOLLA//CA/92037
Journal: BLOOD, %%%1985%%%, V65, N4, P795-802
Language: ENGLISH Document Type: ARTICLE

8/7/106 (Item 2 from file: 434)
DIALOG(R)File 434:SciSearch(R) Cited Ref Sci
(c) 1998 Inst for Sci Info. All rts. reserv.

05369317 Genuine Article#: RJ970 Number of References: 172
Title: IMMUNOASSAY - IS THERE A FUTURE-ROLE FOR NUCLEAR-MEDICINE
Author(s): WITHERSPOON LR
Corporate Source: ALTON OCHSNER MED FDN & OCHSNER CLIN,1514 JEFFERSON
HIGHWAY/NEW ORLEANS//LA/70121; ALTON OCHSNER MED FDN & OCHSNER CLIN/NEW
ORLEANS//LA/70121
Journal: JOURNAL OF NUCLEAR MEDICINE, %%%1983%%%, V24, N10, P952-965
Language: ENGLISH Document Type: REVIEW, BIBLIOGRAPHY

8/7/107 (Item 3 from file: 434)
DIALOG(R)File 434:SciSearch(R) Cited Ref Sci
(c) 1998 Inst for Sci Info. All rts. reserv.

05131114 Genuine Article#: QR133 Number of References: 321
Title: PRODUCT GUIDE FOR RADIOASSAYS AND NON-ISOTOPIC LIGAND ASSAYS
Author(s): EDWARDS L
Journal: CLINICAL CHEMISTRY, %%%1983%%%, V29, N5, P889-986
Language: ENGLISH Document Type: REVIEW, BIBLIOGRAPHY
? ds

Set Items Description
S1 2177 (ANALOG? OR ANALOGUE? OR ANTAGONIST? OR COMPETIT?) AND VIT-
AMIN AND B12
S2 1515 RD S1 (unique items)
S3 49 S2 AND (TRANSCOBALAMIN OR TCII)
S4 62 S2 AND (CANCER? OR PROLIFER?)
S5 57 S4 NOT S3
S6 251 TRANSCOBALAMIN AND ANTIBOD?
S7 121 RD S6 (unique items)
S8 107 S7 AND PY<2000
? logoff y
29may02 12:40:49 User226352 Session D632.3
$7.65 1.365 DialUnits File5
$85.75 49 Type(s) in Format 7
$85.75 49 Types
$93.40 Estimated cost File5
$1.45 0.246 DialUnits File6
$1.45 Estimated cost File6
$20.74 1.213 DialUnits File34
$43.65 9 Type(s) in Format 7
$4.20 1 Type(s) in Format 12
$37.80 9 Type(s) in Format 14
$42.00 10 Type(s) in Format 15
$127.65 29 Types
$148.39 Estimated cost File34
$0.49 0.071 DialUnits File40
$0.49 Estimated cost File40
$0.38 0.079 DialUnits File41
$0.38 Estimated cost File41
$3.84 0.854 DialUnits File50
$34.00 17 Type(s) in Format 7
$34.00 17 Types
$37.84 Estimated cost File50
$3.48 0.927 DialUnits File65
$3.48 Estimated cost File65
$0.45 0.186 DialUnits File68
$0.45 Estimated cost File68
$3.26 0.452 DialUnits File71
$6.40 4 Type(s) in Format 7
$6.40 4 Types
$9.66 Estimated cost File71
$13.39 1.488 DialUnits File73
$62.50 25 Type(s) in Format 7
$62.50 25 Types
$75.89 Estimated cost File73
$1.89 0.370 DialUnits File76
$1.89 Estimated cost File76
$0.30 0.101 DialUnits File77
$2.10 1 Type(s) in Format 7
$2.10 1 Types
$2.40 Estimated cost File77
$2.82 0.806 DialUnits File94
$9.45 7 Type(s) in Format 7
$9.45 7 Types
$12.27 Estimated cost File94
$8.00 3.334 DialUnits File98
$33.50 10 Type(s) in Format 7
$33.50 10 Types
$41.50 Estimated cost File98
$3.09 0.606 DialUnits File103
$3.09 Estimated cost File103
$0.35 0.147 DialUnits File143
$0.35 Estimated cost File143

```
? ds

Set      Items   Description
S1          32   (COBALAMIN OR CBL) (5N) ANTAGONIST?
S2          19   RD S1 (unique items)
? logoff y
       30may02 07:06:45 User226352 Session D633.3
            $0
```

autoimmunity in order to study the pathogenesis of pernicious anemia.

8/7/40 (Item 7 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci
(c) 2002 Inst for Sci Info. All rts. reserv.

05826732 Genuine Article#: XA309 Number of References: 134
Title: Hormone, growth factor, and vitamin handling by proximal tubule cells
Author(s): Christensen EI (REPRINT) ; Birn H
Corporate Source: AARHUS UNIV,INST ANAT, DEPT CELL BIOL/DK-8000 AARHUS C//DENMARK/ (REPRINT)
Journal: CURRENT OPINION IN NEPHROLOGY AND HYPERTENSION, %%%1997%%%, V6, N1 (JAN), P20-27
ISSN: 1062-4821 Publication date: 19970100
Publisher: RAPID SCIENCE PUBLISHERS, 2-6 BOUNDARY ROW, LONDON, ENGLAND SE1 8NH
Language: English Document Type: REVIEW
Abstract: The proximal tubule in the kidney is a main organ for the metabolic clearance of small peptide hormones, and is intensively involved in reabsorption, storage and homeostatic regulation of different vitamins. Recently, the endocytic membrane protein megalin has been shown to bind several smaller peptides, including insulin and epidermal growth factor, as well as to bind and mediate uptake of %%%transcobalamin%%%-B-12, attracting new attention on the cellular mechanisms involved in these processes.

8/7/41 (Item 8 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci
(c) 2002 Inst for Sci Info. All rts. reserv.

05165650 Genuine Article#: VE499 Number of References: 329
Title: COBALAMIN
Author(s): MARKLE HV
Corporate Source: CENTENARY HLTH CTR,2867 ELLESMERE RD/SCARBOROUGH/ON/CANADA/
Journal: CRITICAL REVIEWS IN CLINICAL LABORATORY SCIENCES, %%%1996%%%, V33, N4, P247-356
ISSN: 1040-8363
Language: ENGLISH Document Type: REVIEW
Abstract: Cobalamin (vitamin B-12) is an essential nutrient derived exclusively from bacterial sources. It is an essential cofactor for three known enzymatic reactions. Untreated deficiency, caused by either the autoimmune disease pernicious anemia or nutritional lack, results in a macrocytic anemia and/or subacute combined degeneration of the spinal cord and is eventually fatal. Cobalamin in serum is bound to two proteins, %%%transcobalamin%%% and haptocorrin. The former is responsible for the essential delivery of cobalamin to most tissues. Inadequate tissue availability of cobalamin results in increased concentration of methylmalonic acid and homocyst(e)ine due to inhibition of methylmalonyl-CoA mutase and methionine synthase, respectively. Strict vegetarians have long been known to be at risk of cobalamin deficiency, which develops insidiously over many years. It is now clear that a significant number of the elderly and HIV-positive individuals are also at increased risk of deficiency. Any individual with reduced ability to split cobalamin from food-protein may also become deficient even though intrinsic factor is present. Diagnosis of cobalamin deficiency has frequently relied on total serum cobalamin and the Schilling test. Newer approaches such as analysis of methylmalonic acid, homocyst(e)ine, holotranscobalamin, antiintrinsic factor %%%antibodies%%%, and serum gastrin may provide more cost-effective testing, as well as identify those with a covert deficiency.

8/7/42 (Item 9 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci
(c) 2002 Inst for Sci Info. All rts. reserv.

05027918 Genuine Article#: TH910 Number of References: 0
Title: EPITOPE-SPECIFIC MONOCLONAL-%%%ANTIBODIES%%% (MABS) TO HUMAN %%%TRANSCOBALAMIN%%%-II (TCII) CAN INDUCE APOPTOSIS BY INHIBITING THE CELLULAR UPTAKE OF COBALAMIN (CB1)
Author(s): QUADROS EV; MCLOUGHLIN P; ROTHENBERG SP; MORGAN AC; SHIKORSKAWALKER M; WALKER R
Corporate Source: SUNY HLTH SCI CTR,DIV HEMATOL ONCOL/BROOKLYN//NY/00000; VET ADM MED CTR/BROOKLYN//NY/11209; RECEPTAGEN CORP/EDMONDS//WA/00000; NATL RES COUNCIL,INST BIOL SCI/OTTAWA/ON/CANADA/
Journal: BLOOD, %%%1995%%%, V86, N10 (NOV 15), P489
ISSN: 0006-4971
Language: ENGLISH Document Type: MEETING ABSTRACT

8/7/43 (Item 10 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci
(c) 2002 Inst for Sci Info. All rts. reserv.

04543911 Genuine Article#: TR596 Number of References: 30
Title: IMPAIRED %%%ANTIBODY%%%-RESPONSES TO PNEUMOCOCCAL POLYSACCHARIDE IN ELDERLY PATIENTS WITH LOW SERUM VITAMIN-B-12 LEVELS
Author(s): FATA FT; HERZLICH BC; SCHIFFMAN G; AST AL
Corporate Source: MAIMONIDES HOSP,DEPT MED,4802 10TH AVE/BROOKLYN//NY/11219 ; MAIMONIDES HOSP,DEPT MED/BROOKLYN//NY/11219; SUNY HLTH SCI CTR,DEPT MICROBIOL & IMMUNOL/BROOKLYN//NY/11203
Journal: ANNALS OF INTERNAL MEDICINE, %%%1996%%%, V124, N3 (FEB 1), P299&
ISSN: 0003-4819
Language: ENGLISH Document Type: ARTICLE
Abstract: Objective: To determine whether immunocompetent elderly patients with low serum vitamin B-12 levels have impaired serum %%%antibody%%% responses to the 23-polyvalent pneumococcal polysaccharide vaccine.

Design: Controlled, prospective cohort study.

Measurements: 15 patients with low serum vitamin B-12 levels and 15 age- and diagnosis-matched patients with normal levels were vaccinated. Serum %%%antibody%%% titers to 12 pneumococcal serotypes were measured by radioimmunoassay before and 4 weeks after vaccination.

Results: The difference between the geometric mean of the vaccine anti body titers before a nd after vaccination for all 12 serotypes was lower (P = 0.005) in the patients with low vitamin B-12 levels than in the patients with normal levels. When mean corpuscular volume and age were controlled for, vitamin B-12 remained an independent predictor of %%%antibody%%% response (P = 0.005). Erythrocyte mean corpuscular volume was also an independent predictor of the increase in titer (P = 0.03).

Conclusions: Patients with low vitamin B-12 levels had impaired %%%antibody%%% responses to pneumococcal vaccine. Further study is necessary to determine whether treatment with vitamin B-12 can enhance specific immunoglobulin synthesis and improve the clinical efficacy of the pneumococcal vaccine in patients with low vitamin B-12 levels.

8/7/44 (Item 11 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci
(c) 2002 Inst for Sci Info. All rts. reserv.

03060155 Genuine Article#: MZ689 Number of References: 46
Title: THE TURNOVER OF CO-57-LABELED CYANOCOBALAMIN BOUND TO COBALAMIN BINDING-PROTEINS IN PATIENTS WITH CHRONIC MYELOGENOUS LEUKEMIA
Author(s): GIMSING P; NEXO E
Corporate Source: STRANDORE 13/DK-2100 COPENHAGEN 0//DENMARK/; HERLEV

OCCURRENCE IN A FAMILY OF HIGH SERUM CONCENTRATIONS OF TRANS COBALAMIN II
AUTHOR: KANE S P; HOFFBRAND A V; ALLEN R H; NEALE G
JOURNAL: BR J HAEMATOL 33 (2). 1976 249-259. %%%1976%%%
FULL JOURNAL NAME: British Journal of Haematology
CODEN: BJHEA
RECORD TYPE: Abstract

ABSTRACT: A family is described in which 2 members (a father and daughter),
both with quiescent ulcerative colitis, had abnormally high serum concentrations of a vitamin B12 binding protein. This protein had the molecular weight of %%%transcobalamin%%% II [TC II] on gel filtration, and behaved like TC II with respect to its elution from DE-23 cellulose, its inhibition at acid pH, its absorption by uncoated charcoal, its binding by anti-TC II %%%antibodies%%%, and its ability to transfer vitamin B12 to transformed lymphocytes. Its plasma clearance and tissue distribution when injected into rabbits was indistinguishable from that of TC II from normal subjects. It migrated on electrophoresis in the .beta..gamma. region. This is the 1st case report of related subjects in whom high serum concentrations of TC II were observed.

8/7/34 (Item 1 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci

08071748 Genuine Article#: 243WD Number of References: 35
Title: Cellular import of cobalamin (Vitamin B-12)
Author(s): Seetharam B (REPRINT) ; Bose S; Li N
Corporate Source: MED COLL WISCONSIN,DEPT MED, DIV GASTROENTEROL &
HEPATOL/MILWAUKEE//WI/53226 (REPRINT); MED COLL WISCONSIN,DEPT
BIOCHEM/MILWAUKEE//WI/53226; VET ADM MED CTR,/MILWAUKEE//WI/53226
Journal: JOURNAL OF NUTRITION, %%%1999%%%, V129, N10 (OCT), P1761-1764
ISSN: 0022-3166 Publication date: 19991000
Publisher: AMER INST NUTRITION, 9650 ROCKVILLE PIKE, BETHESDA, MD 20814
Language: English Document Type: ARTICLE
Abstract: Recent studies have isolated and characterized human gastric intrinsic factor (I) and %%%transcobalamin%%% II (TC II) genes, whose products mediate the import of cobalamin (Cbl; Vitamin B-12) across cellular plasma membranes. Analyses of cDNA and genomic clones of IF and TC II have provided some important insights into their sites of expression, structure and function. IF and TC II genes contain the same number, size and position of exons, and four of their eight intron-exon boundaries are identical. In addition, they share high homology in certain regions that are localized to different exons, indicating that IF and TC II may have evolved from a common ancestral gene. Both IF and
TC II mediate transmembrane transport of Cbl via their respective receptors that function as oligomers in the plasma membrane. If-mediated import of Cbl is limited to the apical membranes of epithelial cells; it occurs via a multipurpose receptor recently termed ''cubilin,'' and the imported Cbl is usually exported out of these cells bound to endogenous TC II. On the other hand, TC II-mediated Cbl import occurs in all cells, including epithelial cells via a specific receptor, and the Cbl imported is usually retained, converted to its coenzyme forms, methyl-Cbl and 5'-deoxyadenosyl-Cbl, and utilized.

8/7/35 (Item 2 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci

07934145 Genuine Article#: 226EL Number of References: 93
Title: Receptor-mediated endocytosis of cobalamin (vitamin B-12)
Author(s): Seetharam B (REPRINT)
Corporate Source: MED COLL WISCONSIN,DEPT MED & BIOCHEM, DIV GASTROENTEROL
& HEPATOL/MILWAUKEE//WI/53226 (REPRINT); VET ADM MED CTR,/MILWAUKEE//WI/53226
Journal: ANNUAL REVIEW OF NUTRITION, %%%1999%%%, V19, P173-195
ISSN: 0199-9885 Publication date: 19990000
Publisher: ANNUAL REVIEWS INC, 4139 EL CAMINO WAY, PO BOX 10139, PALO ALTO,
CA 94303-0139
Language: English Document Type: REVIEW
Abstract: Dietary cobalamin (Cbl) (vitamin B-12) is utilized as methyl-Cbl and the coenzyme 5'-deoxyadenosyl Cbl by cells of the body that have the enzymes methionine synthase and methyl malonyl CoA mutase, which convert homocysteine to methionine and methyl malonyl CoA to succinyl CoA, respectively. Prior to conversions and utilizations as the active alkyl forms of Cbl, dietary Cbl is absorbed and transported across cellular plasma membranes by two receptor-mediated events. First, dietary and biliary Cbl bound to gastric intrinsic factor (IF) presented apically to the ileal absorptive enterocytes is transported to the circulation by receptor-mediated endocytosis via apically expressed IF-Cbl receptor. Second, Cbl bound to plasma %%%transcobalamin%%% (TC) II is taken up from the circulation by all cells via a TC II receptor expressed in the plasma membrane of these cells, and in polarized cells via a TC II receptor expressed in the basolateral membranes. This review updates recent work and focuses on (a) the molecular and cellular aspects of Cbl binding protein Ligands, IF and TC II, and their cell-surface receptors, IF-Cbl receptor and TC II receptor; (b) the cellular sorting pathways of internalized Cbl bound to IF and TC II in polarized epithelial cells; and (c) the absorption and transport disorders that cause Cbl deficiency.

8/7/36 (Item 3 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci

06860652 Genuine Article#: ZX229 Number of References: 32
Title: %%%Transcobalamin%%% II and in vitro proliferation of leukemic cells
Author(s): McLean GR; Williams MJ; Woodhouse CS; Ziltener HJ (REPRINT)
Corporate Source: UNIV BRITISH COLUMBIA,BIOMED RES CTR, 2222 HLTH SCI
MALL/VANCOUVER/BC/CANADA/ (REPRINT); UNIV BRITISH COLUMBIA,BIOMED RES
CTR/VANCOUVER/BC/CANADA/
Journal: LEUKEMIA & LYMPHOMA, %%%1998%%%, V30, N1-2 (JUN), P101-109
ISSN: 1042-8194 Publication date: 19980600
Publisher: HARWOOD ACAD PUBL GMBH, C/O STBS LTD, PO BOX 90, READING RG1
8JL, BERKS, ENGLAND
Language: English Document Type: ARTICLE
Abstract: We have recently shown that %%%antibodies%%% to %%%transcobalamin%%% II (TCII) inhibit the in vitro growth of human and
murine leukemic cells. This antiproliferative strategy targets the uptake of cobalamin (cbl), an essential cofactor for two biochemical reactions in humans. To date there has been no appropriate cell culture model available to study antagonism of cbl as a potential antiproliferative strategy. We have established cell culture conditions which allow reproducible measurements of cell proliferation that is dependent on cbl and its carrier protein, TCII. This bioassay has allowed us to demonstrate that several monoclonal %%%antibodies%%%, raised against TCII, are potent inhibitors of cell proliferation and that excess cbl abrogates this inhibitory effect. Thus, supporting our hypothesis that interference with cbl uptake or metabolism will result in inhibition of cell proliferation. Furthermore, cbl metabolism appears to provide a useful target for antiproliferative strategies which now involve the use of inactive cbl analogs. In this review, we update our work on the role of targeting TCII and cbl as an antiproliferative strategy for leukemic cells. We suggest that this strategy may provide a novel direction for anti cancer reagents.

8/7/37 (Item 4 from file: 34)
DIALOG(R)File 34:SciSearch(R) Cited Ref Sci

06849775 Genuine Article#: ZW685 Number of References: 40